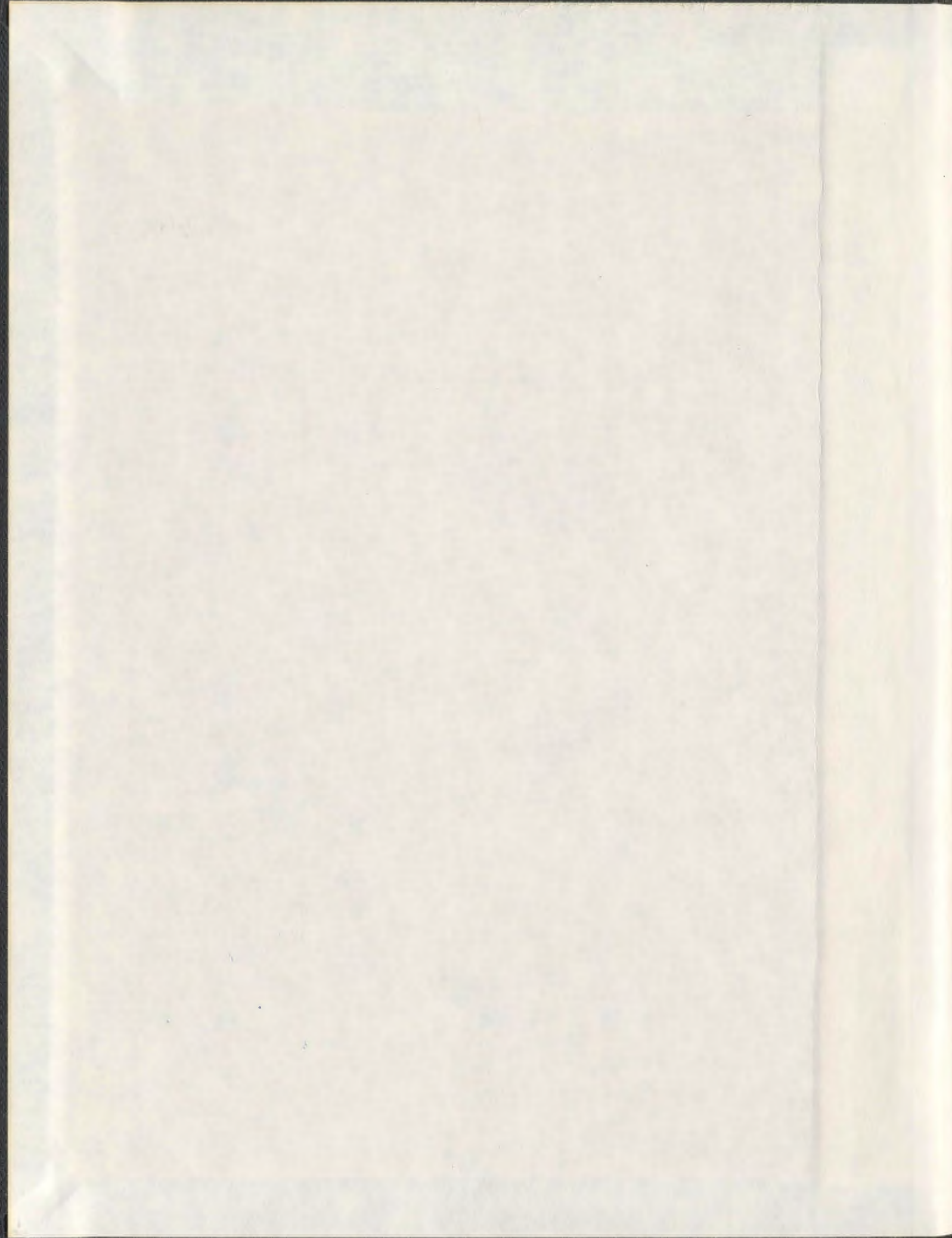


REGULATION OF IRES-MEDIATED TRANSLATION
UNDER PHYSIOLOGICAL AND CELLULAR STRESS CONDITIONS

MARIA LICURSI



**REGULATION OF IRES-MEDIATED TRANSLATION UNDER
PHYSIOLOGICAL AND CELLULAR STRESS CONDITIONS**

by

Maria Licursi

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ABSTRACT

Cap-dependent and IRES-mediated translation initiation mechanisms are regulated differently by cellular machinery. Under conditions such as viral infection, apoptosis and cellular stress, cap-dependent protein synthesis becomes impaired and translation driven by some IRES elements becomes upregulated. The objective of this study was to investigate how cells regulate cap-dependent and IRES-mediated translation in different cellular environments.

To determine whether efficiency of translation initiation by IRES elements differs under normal physiological conditions, we tested 13 bicistronic reporter constructs containing different viral and cellular IRES elements. Bicistronic vectors harboring IRES elements are essential to achieve efficient expression of multiple genes in gene therapy protocols and biomedical applications. The IRES element commonly used in current bicistronic vectors originates from the EMCV and, therefore, was used as standard in this study. The *in vitro* screening in human and mouse fibroblast and hepatocarcinoma cells revealed that the VCIP IRES was the only IRES element that directed translation more efficiently than the EMCV IRES in all cell lines. Furthermore, the VCIP IRES initiated greater reporter expression levels than the EMCV IRES in transfected mouse livers. These results demonstrate that IRES-mediated translation efficiency in physiological conditions is dependent on IRES elements as well as cell types. In addition, this data suggests that VCIP-IRES containing vectors have great potential to improve gene expression and could increase the benefits of bicistronic vectors for experimental and therapeutic purposes.

Viral IRES-mediated translation often remains active when cellular cap-dependent translation is severely impaired under cellular stresses induced by virus infection. Next, we used bicistronic reporter constructs harboring viral IRES elements to investigate how cellular stresses influence the efficiency of viral IRES-mediated translation. Mouse cell line NIH3T3 cells transfected with these bicistronic reporter constructs were subjected to different cellular stresses. Increased translation initiation was observed under amino acid starvation only when EMCV or FMDV IRES elements were present. To identify cellular mechanisms that promoted viral IRES-mediated translation, we investigated the involvement of eukaryotic initiation factor 4E binding protein (4E-BP), general control nondepressed 2 (GCN2) and eukaryotic initiation factor 2B (eIF2B), since these are known to be modulated under amino acid starvation. Knockdown of 4E-BP1 impaired the promotion of EMCV and FMDV IRES-mediated translation under amino acid starvation while GCN2 and eIF2B were not involved. To further investigate how 4E-BP1 regulates translation initiated by EMCV and FMDV IRES elements, we used a phosphoinositide-3 kinase inhibitor (LY294002), an mTOR inhibitor (Torin1) or leucine starvation to mimic 4E-BP1 dephosphorylation induced by amino acid starvation. 4E-BP1 dephosphorylation induced by these treatments was not sufficient to promote the viral IRES-mediated translation. These results suggest that 4E-BP1 regulates EMCV and FMDV IRES-mediated translation under amino acid starvation, but not via its dephosphorylation.

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CO-AUTHORSHIP STATEMENT

All the research work presented in this thesis was primarily performed by the author. That comprised designing the research proposal and protocols in collaboration with the supervisor, performing experiments, collecting and analyzing the data and writing the manuscripts. Most of the work described here has been published in peer-reviewed articles and the references are:

Chapter 3: "*In vitro* and *in vivo* comparison of viral and cellular internal ribosome entry sites for bicistronic vector expression" by Maria Licursi, Sherri L. Christian, Theerawat Pongnopparat and Kensuke Hirasawa, published in *Gene Therapy* (2011) 18, 631–636.

Chapters 4 and 5: "Promotion of viral internal ribosomal entry site mediated translation under amino acid starvation" by Maria Licursi, Yumiko Komatsu, Theerawat Pongnopparat and Kensuke Hirasawa, published in *Journal of General Virology* (2012), 93, 951–962.

I wish to acknowledge the contribution of co-authors: Sherri Christian performed the qRT-PCR assay for Figure 3.4, Theerawat Pongnopparat performed the RT-PCR presented in Figure 3.6 and provided technical assistance for western blot analysis of Figure 5.1, while Yumiko Komatsu performed some of the luciferase assays presented in Figure 4.1.

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LIST OF ABBREVIATIONS

4E-BP	eukaryotic initiation factor 4E binding protein
Apaf-1	apoptotic protease activating factor 1
AQP-4	aquaporin 4
ATCC	American type culture collection
Bcl-2	B-cell lymphoma 2
BIP	binding immunoglobulin protein
BVDV	bovine viral diarrhea virus
c-myc	c-myc proto-oncogene
CAT-1	cationic amino acid transporter 1
CrPV	cricket paralysis virus
CSFV	classical swine fever virus
DMEM	Dulbecco's modified Eagle's medium
dsRNA	double-strand RNA
DTT	dithiothreitol
eIF2	eukaryotic initiation factor 2
eIF2B	eukaryotic initiation factor 2B
eIF3	eukaryotic initiation factor 3
eIF4A	eukaryotic initiation factor 4A
eIF4B	eukaryotic initiation factor 4B
eIF4E	eukaryotic initiation factor 4E
eIF4F	eukaryotic initiation factor 4F

eIF4G	eukaryotic initiation factor 4G
eIF4H	eukaryotic initiation factor 4H
eIF5	eukaryotic initiation factor 5
eIF5B	eukaryotic initiation factor 5B
eIFs	eukaryotic initiation factors
EMCV	encephalomyocarditis virus
FBS	fetal bovine serum
FGF-1	fibroblast growth factor 1
FMDV	foot and mouth disease virus
G3BP	Ras-GTPase-activating protein
GCN2	general control non-depressible 2
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HAV	hepatitis A virus
HCV	hepatitis C virus
HIAP2	inhibitor of apoptosis protein 2
HIF-1 α	hypoxia-inducible factor 1 alpha
hnRNP	heterogeneous nuclear ribonucleoproteins
hnRNP C1	heterogeneous nuclear ribonucleoprotein C1
hnRNP C2	heterogeneous nuclear ribonucleoprotein C2
hnRNP K	heterogeneous nuclear ribonucleoprotein K
hnRNP U	heterogeneous nuclear ribonucleoprotein U

HRI	heme-regulated eIF2 α
HRV	human rhinovirus
HT116	human colorectal carcinoma cell line
Huh7	human hepatoma cell line
IGR	intergenic region
IRES	internal ribosome entry site
ITAF45	IRES trans-acting factor, 45 kDa
ITAFs	IRES trans-acting factors
La autoantigen	lupus erythematosus autoantigen
LPP3	human lipid phosphate phosphohydrolase-3
MEF	mouse embryonic fibroblast
Met-tRNA _i ^{met}	initiator tRNA
MRC5	human lung fibroblast cell line
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTRAQ	mass differential tags for relative and absolute quantification
NIH3T3	murine fibroblast cell line
NRF	human NF-kappaB repressing factor
PA	phosphatidic acid
PABPs	poly(A) binding proteins
PAGE	polyacrylamide gel electrophoresis
PAP2b	phosphatidic acid phosphatase-2b

PCBP2	poly(rc)-binding protein 2
PCR	polymerase chain reaction
PERK	double-stranded RNA-activated protein kinase-like ER kinase
PI3K	phosphoinositide-3 kinase
PKR	dsRNA-dependent protein kinase
PMSF	phenylmethylsulfonyl fluoride
PTB	polypyrimidine tract binding protein
PTV	porcine teschovirus
PV	poliovirus
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative RT-PCR
Rbm3	cold stress-induced mRNA
RT-PCR	reverse transcription polymerase chain reaction
siRNA	small interfering RNA
tRNA	transfer RNA
unr	upstream of n-ras
UPR	unfolded protein response
UTR	untranslated region
VCIP	vascular endothelial growth factor (VEGF) and type 1 collagen inducible protein
VEGF	vascular endothelial growth factor
Vero	monkey kidney epithelium cell line
VSV	vesicular stomatitis virus

XIAP

X-linked inhibitor of apoptosis protein

CHAPTER 1

INTRODUCTION

1.1 Translation initiation mechanisms

Protein translation of eukaryotic mRNAs is comprised of three stages: initiation, elongation, and termination. Of all the steps, initiation is the most tightly regulated, allowing for rapid changes in gene expression in the right intracellular and extracellular environments (Brown & Schreiber, 1996; Gale *et al.*, 2000; Sonenberg *et al.*, 2000). The majority of eukaryotic mRNA translation is initiated by a cap-dependent mechanism while translation of some viral and cellular mRNAs can be initiated by the direct binding of a ribosome to a unique RNA element called an internal ribosome entry site (IRES) (Hellen & Sarnow, 2001; Pelletier & Sonenberg, 1988; Pestova *et al.*, 2001; Stoneley *et al.*, 1998).

1.1.1 mRNA structure

Once the information encoded in DNA has been transcribed, the pre-mRNA molecule has to be processed in the nucleus before it is transported to the cytoplasm as mature mRNA to be translated. The processing of eukaryotic pre-mRNA starts shortly after transcription has been initiated with the addition of a modified guanine nucleotide (7-methylguanosine), known as a cap, to the mRNA 5' end. Capping of mRNA is essential for the process of ribosome recognition in cap-dependent translation initiation as well as mRNA stability, since it protects the mRNA from degradation by RNases (Shatkin *et al.*, 1976). Later, the mRNA molecule is modified in a second process known as splicing which involves the removal of introns. Finally, mRNA is polyadenylated at the 3' end.

The poly(A) tail is important for nuclear export, stability and translation of mRNA (Kahvejian *et al.*, 2001; Wells *et al.*, 1998).

1.1.2 Cap-dependent translation initiation

Cap-dependent translation is initiated by the recognition of the cap structure at the 5' end of the mRNA by the eukaryotic initiation factor 4E (eIF4E). This forms the eukaryotic initiation factor 4F (eIF4F) complex with the RNA helicase eukaryotic initiation factor 4A (eIF4A) and the scaffold protein eukaryotic initiation factor 4G (eIF4G) (Figure 1.1) (Gebauer & Hentze, 2004; Shatkin, 1985). The binding of the eIF4F complex to the cap structure facilitates the formation of a closed loop of mRNA by the interaction of poly(A) binding proteins (PABPs) with eIF4G (Imataka *et al.*, 1998; Tarun & Sachs, 1996). This mRNA conformation is required to stimulate efficient translation (Kahvejian *et al.*, 2005; Munroe & Jacobson, 1990). The eIF4F complex guides the activated 40S subunit of the ribosome, which contains the ternary complex (Met-tRNA_i^{Met}-eIF2-GTP), to the 5' end of the mRNA through its association with the eukaryotic initiation factor 3 (eIF3) (Sonenberg *et al.*, 2000). This complex travels along the 5' untranslated region (UTR) of the mRNA in a process known as scanning until it reaches the first AUG codon in the appropriate context, known as the Kozak sequence (Kozak, 1989). The consensus sequence that is most favorable for a start codon is gccRccAUGG, where R is any purine, upper-case letters indicate highly-conserved bases, and lower-case letters indicate the most common base present at those positions. This scanning is facilitated by the

Figure 1.1: Schematic representation of cap-dependent translation initiation.

Figure shows a simplified diagram of ribosome recruitment to mRNA by cap-dependent mechanism that only includes the main eukaryotic initiation factors (eIFs) discussed in this study. Cap-dependent translation begins with the formation of the eIF4F complex (eIF4E, eIF4G and eIF4A), which binds to the cap structure (cap) in the 5' end of the mRNA. The mRNA is circularized by interaction of poly(A) binding proteins (PABPs) with eIF4G. The 43S complex (40S ribosome subunit, eIF3 and ternary complex) binds to eIF4G through eIF3. This complex scans the mRNA 5' UTR until the first AUG codon. Recognition of the start codon and GTP hydrolysis lead to dissociation of eIFs and joining of the 60S ribosomal subunit.

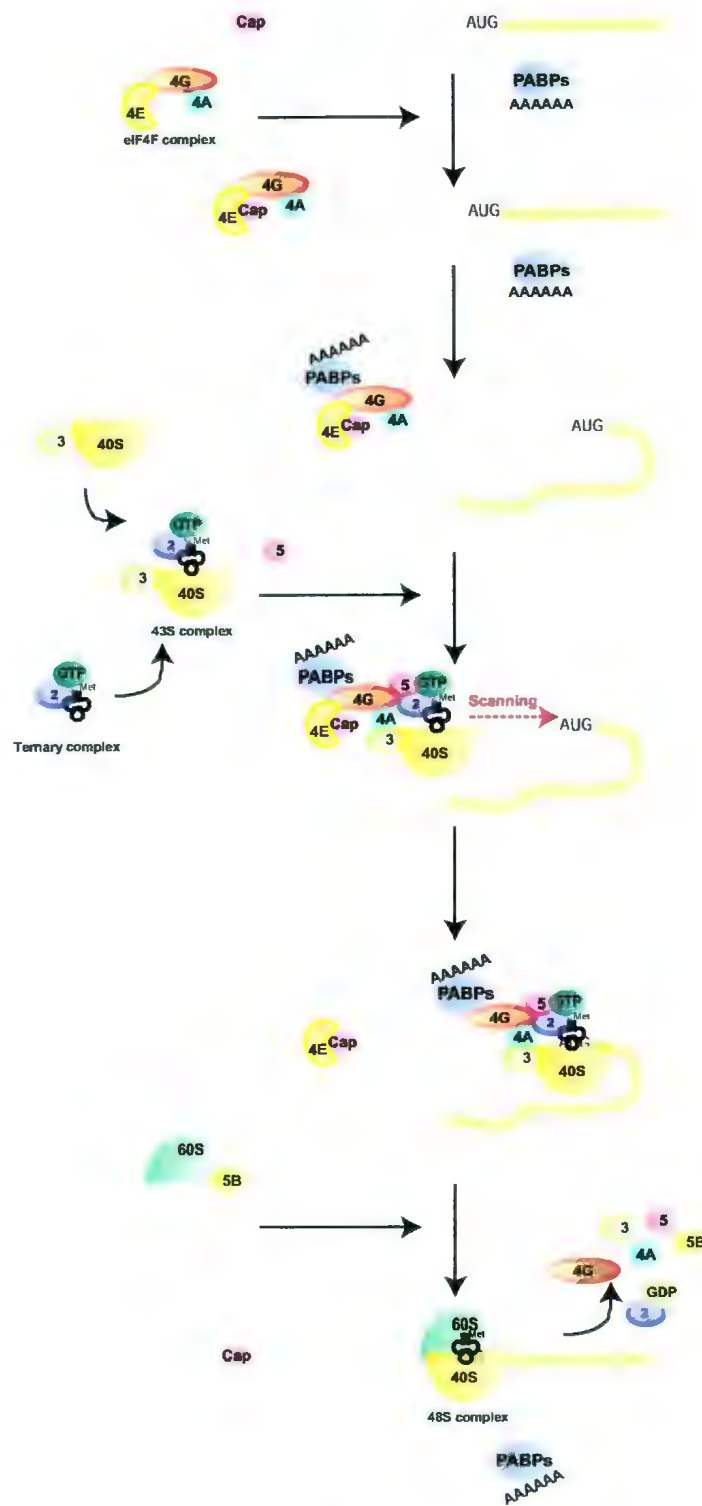


Figure 1.1

eukaryotic initiation factors 4B (eIF4B) and 4H (eIF4H), which enhance eIF4A helicase activity in an ATP dependent manner. Once the Met-tRNA_i^{Met} anticodon pairs with the AUG codon, the eukaryotic initiation factor 5 (eIF5) promotes the GTP hydrolysis of eukaryotic initiation factor 2 (eIF2)-GTP. This results in the dissociation of eIF2-GDP and other eukaryotic initiation factors (eIFs). Finally, the eukaryotic initiation factor 5B (eIF5B) mediates the joining of the 60S ribosomal subunit to the 80S ribosome, initiating the elongation phase (Gebauer & Hentze, 2004).

1.1.3 IRES-mediated translation initiation

In certain circumstances, such as the highly structured 5' UTR mRNA, protein translation by the scanning mechanism is not possible. Several cellular and viral mRNAs are translated instead by an alternative mechanism, through an IRES element (Hellen & Sarnow, 2001; Pelletier & Sonenberg, 1988; Pestova *et al.*, 2001; Stoneley *et al.*, 1998). These IRES elements were first discovered in the mRNAs of the *Picornaviridae* (Jang *et al.*, 1988; Pelletier & Sonenberg, 1988). Since then, several other viral and cellular mRNAs have been reported to contain IRES elements (Fernandez *et al.*, 2001; Holcik *et al.*, 1999; Johannes & Sarnow, 1998; Macejak & Sarnow, 1991; Stoneley *et al.*, 1998). The IRES contains a high degree of RNA secondary structure and recruits the 40S ribosomal subunit in close proximity to the initiation codon in a cap-independent manner (Figure 1.2). Most of the eukaryotic initiation factors involved in cap-dependent translation are also implicated in IRES-mediated translation (Hellen & Sarnow, 2001). In addition, cellular proteins known as IRES *trans*-acting factors (ITAFs) are also required

for efficient IRES-mediated translation (Komar & Hatzoglou, 2005; Martinez-Salas *et al.*, 2001). More than two decades ago, a nucleotide sequence study of the picornavirus demonstrated that the 5' UTR was around 500 nucleotides in length and was highly structured, suggesting that it was incompatible with conventional scanning mechanisms. In addition, picornaviruses possess a single-stranded, positive sense RNA which contains multiple AUG non-initiating codons and lacks the cap structure at 5' end which is essential for cap-dependent translation (Jackson *et al.*, 1990; Jang *et al.*, 1990). Although there are some common primary sequences or secondary structures conserved between certain viral IRES elements belonging to the same family, nonrelated IRES elements have no similarities in sequence or structure. Additionally, the subset of ITAFs that regulates translation initiation appears to be specific to each IRES element (Komar & Hatzoglou, 2005). For example, different expression levels of specific ITAFs required for efficient viral IRES-mediated translation may influence tissue tropism (Gromeier *et al.*, 2000; Pilipenko *et al.*, 2000).

1.1.3.1 Viral IRES

IRES-mediated translation is advantageous for viruses harboring an IRES since the virus can continue to efficiently generate viral proteins even while protein synthesis by cap-dependent translation has been compromised during cellular stress or apoptosis. Viruses containing IRES elements belong almost exclusively to *Picornaviridae*, *Flaviviridae* and *Dicistroviridae* families. Based on IRES sequence homology, and their requirements for efficient translation, viral IRES elements have been classified into different groups. While

some primary sequences and specially secondary structures are conserved among viral IRESs belonging to the same group, conservation among the different groups is very poor (Jackson *et al.*, 1994). The requirements for canonical initiation factors, as well as specific ITAFs, also differ among the IRES groups (Borman *et al.*, 1995).

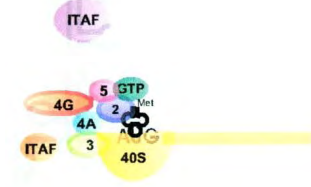
1.1.3.1.1 Picornavirus IRES

Picornaviral IRESs have been classified into four groups (I-IV). IRES elements from the enteroviruses and rhinoviruses belong to type I, whose prototypes are the poliovirus and the human rhinovirus, respectively. The Type II IRES group includes viruses belonging to the genus cardioviruses and aphthoviruses. examples of which include the encephalomyocarditis virus (EMCV) and the foot and mouth disease virus (FMDV), respectively. In addition, the hepatitis A virus (HAV) IRES belongs to the type III group, while the porcine teschovirus (PTV) is part of the type IV IRES group.

The IRES elements from types I, II, and III have a similar RNA structure with a central core and a cloverleaf structure resembling a tRNA molecule (Jackson *et al.*, 1994; Pilipenko *et al.*, 1989). Located at the 3' end of the IRES element is a pyrimidine-rich tract, and about 25 nucleotides downstream an AUG codon, responsible for the recruitment of translational machinery, can be found (Kaminski *et al.*, 1990; Lopez de Quinto & Martinez-Salas, 2000; Pelletier & Sonenberg, 1988). Type II IRES elements can initiate translation directly at the AUG within the starting point (Figure 1.2B), although FMDV seems to be able to translate more efficiently from a second AUG

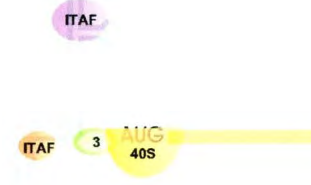
Figure 1.2: Schematic representation of IRES-mediated translation initiation by different type of IRES elements. **A)** Model of Picornavirus Type I IRES which requires all eIFs with the exception of eIF4E. Translation is initiated at an AUG codon placed downstream of the IRES, reached by a scanning mechanism. **B)** Model of Picornavirus Type II IRES is similar to Picornavirus Type I IRES but is able to initiate translation directly at the AUG where ribosome is recruited. **C)** Model of HCV-like IRES only requires eIF3, eIF2 and the ternary complex for recruitment of ribosome 40S subunit. **D)** Model of Dicistrovirus IRES in which ribosome binds directly to the IRES element without requirement of any eIFs.

B



Picornaviruses Type II IRES

D



Dicistrovirus IRES

10

located 84 nucleotides downstream (Belsham, 1992). For type I IRES elements, the AUG codon placed at the entry site is silent during translation and the authentic AUG initiation codon is downstream, separated by a variable non-conserved sequence (Figure 1.2A). However, it remains unclear if the initiation codon is reached by scanning processes or whether the ribosome subunit skips part of the sequence (Jang *et al.*, 1990; Pestova *et al.*, 1994). The Translation initiation of type I and II IRES elements require the whole 43S complex (eIF2, eIF3 and a 40S ribosomal subunit), as well as eIF4G, whose binding to the IRES is stimulated by the RNA helicase eIF4A (Pestova *et al.*, 1996b). In addition to these canonical initiation factors, HAV IRES, which belongs to type III, requires an intact cap-binding complex, which includes eIF4E, for efficient translation (Ali *et al.*, 2001).

The latest type of picornavirus IRES includes members of the theschovirus genus. Interestingly, there are no obvious sequence or structure similarities with IRESs from other picornaviruses. Furthermore, PTV IRES shares structural and functional features with the hepatitis C virus (HCV) IRES. For that reason, it is sometimes classified as HCV-like IRES. In contrast to other picornaviruses, PTV only requires a ternary complex with eIF3 to bind the 40S subunit to the IRES and initiate translation (Pisarev *et al.*, 2004) (Figure 1.2C).

1.1.3.1.2 Flavivirus IRES

The prototype IRES element from *Flaviviridae* family is the HCV IRES (Tsukiyama-Kohara *et al.*, 1992). This group also includes IRES elements from the pestivirus, such as

bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV). These IRES elements differ in structure and molecular mechanism from picornavirus IRESs. The HCV IRES comprises domains II-IV as well as some nucleotides from the 5' proximal coding regions (Fletcher & Jackson, 2002; Reynolds *et al.*, 1995). In contrast to picornaviral IRES elements, the 40S ribosomal subunit can directly bind to the HCV IRES without any initiation factors. A ternary complex (eIF2-GTP-Met-tRNA_i^{Met} and eIF3) is required for the assembly of the 80S ribosome (Figure 1.2C) (Pestova *et al.*, 1998).

1.1.3.1.3 Dicistrovirus IRES

Invertebrate viruses belonging to *Dicistroviridae* family possess the simplest IRES driven mechanism for translation initiation. Cricket paralysis virus (CrPV) is the prototype of this group. In this case, the IRES element is located between two open reading frames in the intergenic regions (IGR) of the virus. The ribosomal 40S subunit binds directly to the IRES without the requirement of any of the canonical initiation factors (Figure 1.2D) (Pestova *et al.*, 1996a; Wilson *et al.*, 2000).

1.1.3.2 Cellular IRES

Shortly after the discovery of IRES-mediated translation in certain viruses, an IRES element was identified in the 5' UTR of the binding immunoglobulin protein (BIP)

mRNA (Macejak & Sarnow, 1991). Many others cellular IRESs were discovered subsequently and currently there are more than one hundred cellular mRNAs reported to contain IRES elements (Mokrejs *et al.*, 2009). While viral IRES elements have a secondary structure, which is phylogenetically conserved in related viruses, there is no evident conservation of RNA structure between cellular IRES elements. Furthermore, while deletions or mutational analysis in viral IRES elements have been shown to abrogate translation initiation, similar studies in cellular IRES show that they only have a partial effect on protein synthesis (Le Quesne *et al.*, 2001; Stoneley *et al.*, 1998; Yang & Sarnow, 1997). Since cellular mRNAs harboring IRES elements are also capped, protein translation of genes such as the c-myc proto-oncogene (c-myc) can be initiated by cap-dependent translation (Andreev *et al.*, 2009; Stoneley *et al.*, 2000). However, due to the long and highly structured 5' UTR it is believed that scanning process is not very efficient. Currently, the mechanisms that regulate the IRES-mediated translation of cellular IRES remains to be clarified. In addition, although the list of cellular IRESs continues growing, problems in the validation of putative cellular IRES elements have raised some concerns (Kozak, 2001; 2005). The presence of cryptic promoters or spurious splicing events was found to be responsible for the translational activity of some mRNAs that had been reported to contain an IRES element. For that reason, it is indispensable to test potential IRES element by performing rigorous and reliable controls (Holcik *et al.*, 2005; Van Eden *et al.*, 2004a).

1.1.3.3 IRES Trans-acting factors (ITAFs)

Beside canonical initiation factors, other cellular proteins known as ITAFs are required for efficient IRES-mediated translation (Komar & Hatzoglou, 2005; Martinez-Salas *et al.*, 2001). The subset of ITAFs that regulates translation initiation appears to be specific to each IRES element (Komar & Hatzoglou, 2005). The specific function of these auxiliary factors is not completely solved. Though it has been suggested that ITAFs can modulate IRES activity either by modifying the IRES secondary structure or by facilitating the recruitment of initiation factors (Komar & Hatzoglou, 2005; Lewis & Holcik, 2008; Pilipenko *et al.*, 2000; Spriggs *et al.*, 2008).

Several of the ITAFs involved in IRES-mediated translation belong to the heterogeneous nuclear ribonucleoprotein (hnRNP) family, which plays roles in a variety of cellular functions such as pre-mRNA processing and mRNA export, stability, and translation (Dreyfuss *et al.*, 2002). One striking feature of hnRNP proteins is that they shuttle between the nucleus and the cytoplasm (Komar & Hatzoglou, 2005; Lewis & Holcik, 2008; Spriggs *et al.*, 2005). It has been reported that changes in the subcellular localization of these ITAFs influences IRES-mediated translation. The list of ITAFs that have been reported to bind viral or cellular IRES includes, among others, proteins such as the lupus autoantigen (La), polypyrimidine tract binding protein (PTB), ITAF45 (Ebp1), heterogeneous nuclear ribonucleoprotein C1 and C2 (hnRNPC1/C2), poly(rC) binding protein 2 (PCBP2), and upstream of n-ras (unr) (Holcik *et al.*, 2003; Meervitch *et al.*, 1993; Mitchell *et al.*, 2005; Pilipenko *et al.*, 2000; Schepens *et al.*, 2007; Walter *et al.*, 1999). Additional IRES binding proteins have been recently identified by RNA affinity

chromatography and mass spectrometry (Lewis *et al.*, 2007; Pacheco & Martinez-Salas, 2010; Pacheco *et al.*, 2008). Although the ITAFs can interact with IRES elements, functional analyses are required to validate their functional roles in facilitating IRES-mediated translation.

1.1.3.4 The use of IRES elements in expression vectors

Gene co-expression can be obtained through several different approaches (Figure 1.3). The first option is to use two independent vectors for each gene of interest (Figure 1.3A). However, the delivery of both vectors into the same cell for simultaneous gene expression is very inefficient. The second alternative involves the delivery of a single vector with different promoters to direct the transcription of each gene of interest (Figure 1.3B). The main caveat of this approach is a phenomenon that causes promoter silencing that may result in the loss of expression of one of the genes over time (Allera-Moreau *et al.*, 2006; Allera-Moreau *et al.*, 2007; Delluc-Clavieres *et al.*, 2008; Li *et al.*, 2007; Morgan *et al.*, 1992). Although this is not a disadvantage for transient transfections, simultaneous gene expression might be compromised in the long term. The third approach is based on the use of bicistronic or multicistronic vectors with an IRES element, which is used as a linker between genes, allowing gene co-expression from a single transcript unit (Bouabe *et al.*, 2008; Martinez-Salas, 1999; Wang *et al.*, 2005b; Wong *et al.*, 2002) (Figure 1.3C). This strategy guarantees the delivery of genes of interest in the cell, avoiding the problem of promoter suppression. Because of their ability to produce multiple proteins in a single

Figure 1.3: Schematic representation of different approaches for gene co-expression.

A) Gene co-expression approach using single vectors containing each gene of interest. **B)** Gene co-expression approach using a single vector containing each gene of interest under different promoters. **C)** Gene co-expression approach using a multicistronic vector in which an IRES element is utilized to drive translation of a second gene of interest. Cells expressing "gene A" are represented in blue, cells expressing "gene B" are represented in yellow and those cells expressing both genes are represented in green.

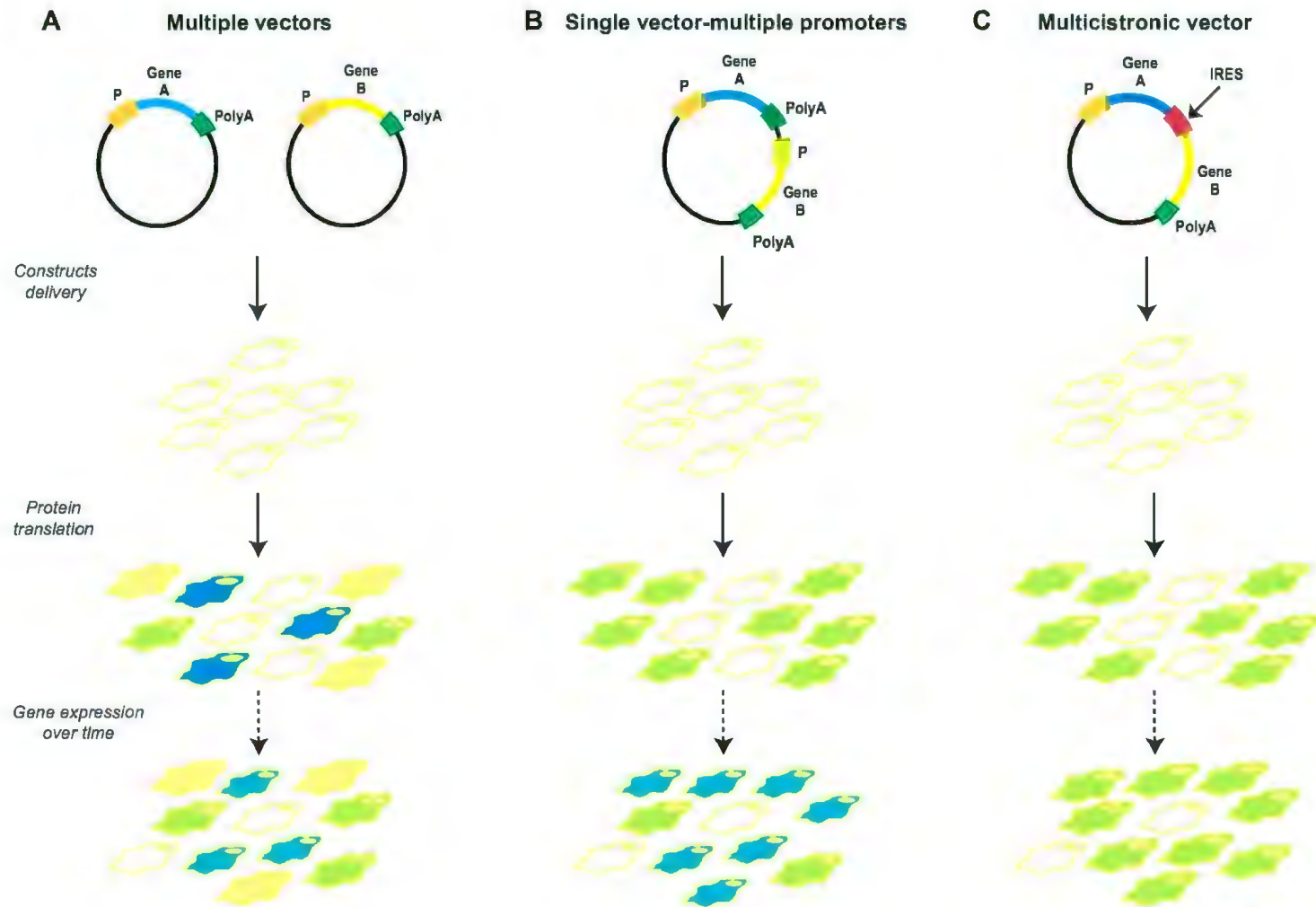


Figure 1.3

cell, bicistronic vectors have been widely used in the field of gene therapy and biomedical research (Delluc-Clavieres *et al.*, 2008; Gallardo *et al.*, 1997; Ngoi *et al.*, 2004; Wagstaff *et al.*, 1998). At present time, the IRES element from EMCV is the most commonly used in vector design. Bicistronic vectors containing EMCV IRES have been widely used for *in vitro* and *in vivo* applications such as co-expression of reporter genes or selective markers, generation of induced pluripotent stem cells in biomedical research as well as treatment of complex disorders in gene therapy protocols (Albagli-Curiel *et al.*, 2007; Delluc-Clavieres *et al.*, 2008; Gallardo *et al.*, 1997; Ngoi *et al.*, 2004; Wagstaff *et al.*, 1998). The complete EMCV sequence was reported in 1986 (Palmenberg *et al.*, 1984), and shortly later its 5'UTR was shown to initiate protein translation of the downstream gene by a cap-independent mechanism in a bicistronic reporter gene construct (Jang *et al.*, 1988). This discovery encouraged the development of the first commercial vector including the EMCV IRES element, pCITE1, by Novagen (Madison, WI, USA) and since then several vectors containing the EMCV IRES to allow expression of a second gene have been engineered. However, adequate expression level of gene downstream of EMCV IRES is not always achieved (Li *et al.*, 2007; Mizuguchi *et al.*, 2000; Wang *et al.*, 2005b; Wong *et al.*, 2002). To solve this, alternative cellular and viral IRES elements, which initiate protein expression more efficiently, have been searched (Allera-Moreau *et al.*, 2007; Bernstein *et al.*, 1997; Borman *et al.*, 1997; Douin *et al.*, 2004; Fux *et al.*, 2004; Harries *et al.*, 2000; Hennecke *et al.*, 2001; Ramesh *et al.*, 1996; Wong *et al.*, 2002). Several cellular IRES such as eIF4G (Wong *et al.*, 2002), BIP (Wong *et al.*, 2002), c-myc (Wong *et al.*, 2002), VEGF (Wong *et al.*, 2002) and fibroblast growth factor 1 (FGF-1)

(Delluc-Clavieres *et al.*, 2008) IRES have been reported to demonstrate higher efficiency than EMCV IRES.

1.2 Translation under cellular stress conditions

Cells respond to intracellular or extracellular stress through a number of mechanisms designed to maintain or recover homeostasis. These key cellular pathways are known as stress response pathways and include a variety of cascades that are activated by different stress conditions that result in selective gene transcriptional activation to promote cell survival or, in case the damage is too severe, lead to cell death (Kultz, 2005). Stress response pathways include: heat shock response, oxidative stress response, unfolded protein response (UPR, also called ER stress response) and DNA damage response. Heat shock response pathway is activated by mild heat stress and heavy metals, and a group of genes known as heat shock proteins are upregulated (Morimoto *et al.*, 1996; Westerheide & Morimoto, 2005). In response to the presence of reactive oxygen species (ROS) produced by several environmental toxics or cellular reactions, the cell activates the oxidative stress response (Trachootham *et al.*, 2008). In addition, cellular conditions like glucose starvation, hypoxia or inhibition of protein glycosylation cause accumulation of unfolded proteins in the endoplasmic reticulum and in turn activate the UPR with the subsequent upregulation of chaperones such as BIP (Bertolotti *et al.*, 2000; Schroder, 2008). Finally, cells exposed to chemotherapeutic agents, or genotoxic agents like ultraviolet (UV) light, respond by the DNA damage response that activates p53 which induces transcriptional activation of several proteins involved in DNA repair processes

(Christmann *et al.*, 2003; Harper & Elledge, 2007). Regardless of the type of cellular stress, global protein synthesis is halted after stress response pathway activation. Stress induced inhibition of protein translation is caused by either regulation of mTOR downstream target 4E-BPs or by eIF2 α phosphorylation. However, it has been reported that a selective group of mRNA that encode for proteins involved in cell survival or apoptosis, remains bound to polysomes. Interestingly, several of these mRNAs have been shown to contain an IRES element.

1.2.1 Cap-dependent translation inhibition during cellular stress

Cellular stresses often lead to the impairment of cap-dependent translation, induction of apoptosis, inhibition of DNA synthesis, and cell cycle arrest (Clemens, 2001; Pearce & Humphrey, 2001). The inhibition of cap-dependent translation is usually caused by changes in the availability of the eIF4F complex or the ternary complex. The formation of the eIF4F complex is essential for translation initiation by a cap-dependent mechanism, since eIF4E recognizes the cap at the 5' end of the mRNA. Eukaryotic initiation factor 4E binding proteins (4E-BPs) compete with eIF4G for the binding site on eIF4E, modulating eIF4F complex formation (Figure 1.4A) (Pause *et al.*, 1994). Under normal conditions, an active mammalian target of the rapamycin (mTOR) pathway maintains 4E-BPs phosphorylation and promotes cap-dependent translation. When the mTOR pathway is inhibited due to cellular stress, 4E-BPs undergo dephosphorylation and bind tightly to eIF4E. Since eIF4E bound to 4E-BPs is unable to interact with the eIF4G scaffold

Figure 1.3: Schematic representation of different approaches for gene co-expression.

A) Gene co-expression approach using single vectors containing each gene of interest. **B)** Gene co-expression approach using a single vector containing each gene of interest under different promoters. **C)** Gene co-expression approach using a multicistronic vector in which an IRES element is utilized to drive translation of a second gene of interest. Cells expressing "gene A" are represented in blue, cells expressing "gene B" are represented in yellow and those cells expressing both genes are represented in green.

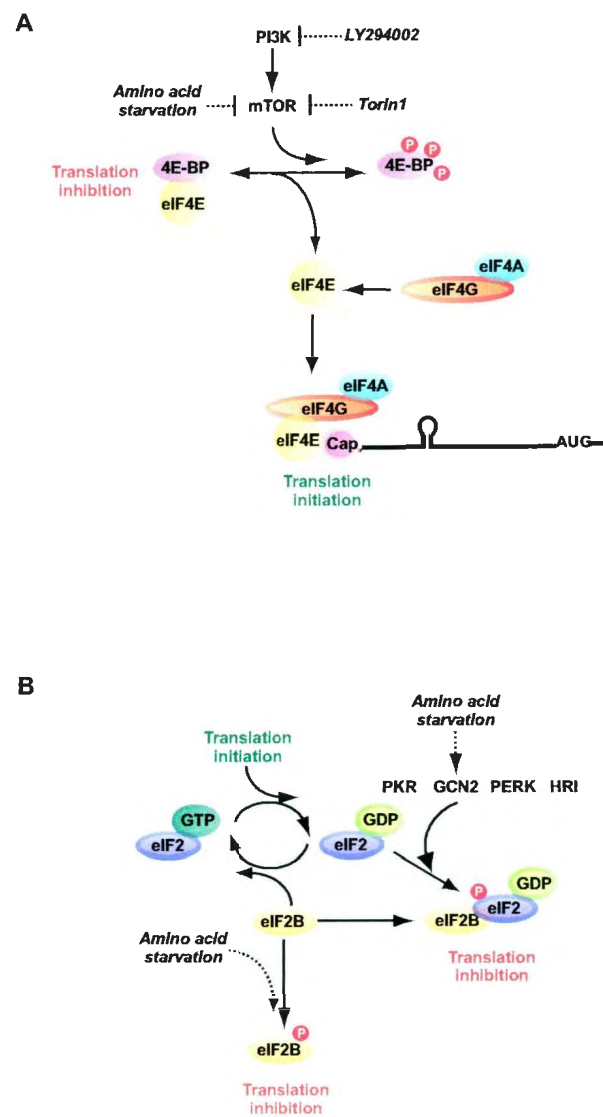


Figure 1.4

component of the eIF4F complex, cap-dependent translation is suppressed. The second mechanism that is tightly regulated is the formation of the ternary complex (eIF2-GTP-Met-tRNA_i^{Met}). GTP-bound eIF2 is essential for the association of Met-tRNA_i^{Met} with the 40S ribosomal subunits (Pain, 1996; Proud, 2005). At the end of translation initiation, eIF2 is released as eIF2-GDP, which is continuously recycled to eIF2-GTP by the catalytic activity of the eukaryotic initiation factor 2B (eIF2B) (Figure 1.4B) (Kimball, 1999; Proud, 1992). Different cellular stresses regulate the availability of the ternary complex by inducing phosphorylation on the serine 51 of the eIF2 α subunit (Gebauer & Hentze, 2004). The phosphorylated eIF2 α sequesters eIF2B and thus inhibits the initiation of cap-dependent translation (Deng *et al.*, 2002; Hershey, 1991). There are four known kinases (general control nondepressible 2 (GCN2), heme-regulated eIF2 α kinase (HRI), double-stranded RNA-activated protein kinase-like ER kinase (PERK), and dsRNA-dependent protein kinase (PKR)) that modulate the phosphorylation of eIF2 α in response to certain cellular stresses (Kimball, 2001; Price & Proud, 1990; Wek *et al.*, 2006).

In addition to the translation regulation described above, which relies on changes of initiation factors phosphorylation status, translation initiation is also modulated by the proteolysis of the scaffold protein eIF4G. During a picornaviral infection, eIF4G undergoes irreversible cleavage by viral proteases. The eIF4G proteolysis separates the domain of eIF4G, that binds to eIF4E, from the rest of the scaffold protein, resulting in the shutoff of the host's protein synthesis (Lloyd, 2006; Schneider & Mohr, 2003), while viral proteins can still be efficiently translated by the IRES-mediated mechanism. During

apoptosis, eIF4G also undergoes cleavage caused by caspases, which in turn causes the impairment of cap-dependent protein translation (Bushell *et al.*, 2000; Clemens *et al.*, 2000; Marissen *et al.*, 2000).

1.2.2 Cellular IRES-dependent translation upregulation during cellular stress

Under cellular stress conditions, cap-dependent protein synthesis is impaired while translation driven by IRES elements is often maintained or even upregulated (Stein *et al.*, 1998; Stoneley & Willis, 2004; Subkhankulova *et al.*, 2001). This feature of IRES-mediated translation has led to the belief that cellular mRNAs containing IRES elements may play a critical role in maintaining cellular homeostasis or inducing apoptosis under cellular stress conditions (Holcik, 2003; Yamasaki & Anderson, 2008). Several studies have shown efficient IRES-mediated translation from cellular mRNAs under different stress conditions (Chappell *et al.*, 2001; Nevins *et al.*, 2003a; Pyronnet *et al.*, 2000; Van Eden *et al.*, 2004b). For example, amino acid starvation increases translation initiated by the cationic amino acid transporter 1 (CAT-1) IRES element in C6 rat glioma cells while cap-dependent translation becomes severely impaired (Fernandez *et al.*, 2001). The translation initiation of certain cellular IRES elements has also been shown to be upregulated under other cellular stress conditions such as hypoxia for vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1 alpha (HIF-1 α) IRES (Akiri *et al.*, 1998; Lang *et al.*, 2002; Stein *et al.*, 1998), endoplasmic reticulum stress for inhibitor of apoptosis protein 2 (IAP2) IRES (Van Eden *et al.*, 2004b), mitosis for B-cell lymphoma 2 (Bcl-2) IRES (Sherrill *et al.*, 2004), apoptosis for apoptotic protease activating factor 1

(Apaf-1) and X-linked inhibitor of apoptosis protein (XIAP) IRES (Holcik *et al.*, 2003; Holcik & Korneluk, 2000; Mitchell *et al.*, 2001; Mitchell *et al.*, 2003), and heat shock for BIP IRES (Kim & Jang, 2002; Macejak & Sarnow, 1991).

Studies that perform polysome profiling following cellular stress show that mRNAs representing approximately 3-5% of the genome remain associated with the polysomes (Holcik & Sonenberg, 2005; Johannes *et al.*, 1999). Interestingly, an IRES element was found in the majority of these mRNAs. In addition, these studies have demonstrated that, under certain stresses, a different subset of mRNAs is translated, with no overlap (Spriggs *et al.*, 2008). Thus, it was suggested that approximately 10-15% of all cellular mRNAs are predicted to be able to translate through an IRES element (Mitchell *et al.*, 2005; Spriggs *et al.*, 2008).

1.2.3 Translational regulation during amino acid starvation

Amino acid starvation is one of the cellular stresses that are known to modify the rate of cap-dependent and cellular IRES-mediated translation (Fafournoux *et al.*, 2000; Hara *et al.*, 1998; Kilberg *et al.*, 1994). It has been reported that after amino acid starvation, cap-dependent protein translation is suppressed through three different pathways: mTOR (Kimball, 2001), GCN2 (Harding *et al.*, 2000), and eIF2B (Wang & Proud, 2008) (Figure 1.4). Under amino acid starvation, 4E-BPs undergo dephosphorylation and bind tightly to eIF4E. Since eIF4E is not able to interact with eIF4G to form initiation complexes while bound to 4E-BPs, cap-dependent translation is repressed (Figure 1.4A). On the contrary,

the presence of amino acid leads to a multisite phosphorylation of 4E-BPs, causing eIF4E to be released. This results in the promotion of cap-dependent translation initiation. When a cell is depleted of amino acids, the protein kinase GCN2 is activated by the accumulation of uncharged tRNAs, which phosphorylates the alpha subunit of eIF2, suppressing cap-dependent translation as a result (Fernandez *et al.*, 2002; Kimball, 2001) (Figure 1.4B). The phosphorylation of eIF2 α reduces the dissociation rate of eIF2 from eIF2B and inhibits the recycling of the inactive GDP-bound eIF2 into a translational active GTP-bound eIF2 (Rowlands *et al.*, 1988). In addition, amino acid starvation has been shown to inhibit eIF2B activity directly by modulating one of its subunits, eIF2B ϵ (Wang & Proud, 2008).

1.3 Thesis objectives

We have started to understand that cellular mRNAs containing an IRES element play critical roles in maintaining cellular homeostasis. Moreover, viral IRES-mediated translation is also an advantage for viruses, such as picornaviruses and HCV, to efficiently produce viral proteins under cellular stress conditions. When cells are under stress conditions, they activate the cellular stress response pathways, which are known to suppress cap-dependent translation. However, it still remained to be studied whether/how these pathways regulate IRES-mediated translation. *The objective of this thesis is to clarify how cells regulate IRES-mediated translation under different cellular environments.* I will determine whether efficiency of translational initiation by IRES

elements differs under normal physiological conditions (Chapter 3). I will also determine whether cellular stresses modulate efficiency of translation initiation by viral IRES elements (Chapter 4). Finally, I will identify cellular machineries involved in promotion of viral IRES-mediated translation under stress conditions (Chapter 5).

CHAPTER 2
MATERIALS AND METHODS

2.1 Constructs

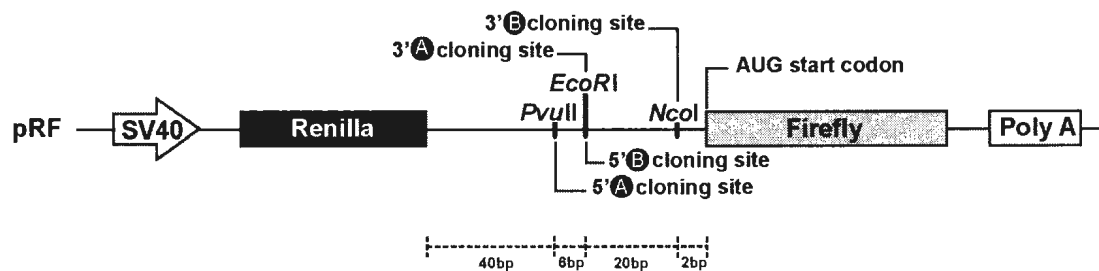
Five viral and eight cellular IRES elements were used in this study: FMDV (Lafuente *et al.*, 2002), HCV (Lafuente *et al.*, 2002), EMCV (Johansen & Morrow, 2000), poliovirus (PV) (Johansen & Morrow, 2000), human rhinovirus (HRV) (Stoneley *et al.*, 1998), cold stress-induced mRNA (Rbm3) (Chappell *et al.*, 2001), human NF-kappaB repressing factor (NRF) (Oumard *et al.*, 2000), Apaf-1 (Holcik *et al.*, 2003), BIP (Nevins *et al.*, 2003b), vascular endothelial growth factor (VEGF) and type 1 collagen inducible protein (VCIP) (Blais *et al.*, 2006), aquaporin 4 (AQP-4) (Baird *et al.*, 2007), c-myc (Nevins *et al.*, 2003b) and CAT-1 (Fernandez *et al.*, 2001) (Table 2.1). Sequence information of the IRES elements is included in Table 2.1. The reporter constructs were prepared by inserting an IRES element into the bicistronic pRF vector (Figure 2.1). The pRF bicistronic constructs containing the HRV IRES and Rbm3 IRES were obtained from Dr. A. Willis and Dr. V.P. Mauro respectively (Chappell *et al.*, 2001; Stoneley *et al.*, 1998). IRES elements from EMCV, FMDV, HCV, PV, AQP-4 and NRF were amplified from the original plasmid by PCR using specific primers (Table 2.2). Apaf-1, BIP, c-myc, and VCIP IRES fragments were digested from the original constructs and then subcloned into the pRF bicistronic construct. For RNA affinity chromatography, FMDV IRES was PCR amplified from pRF bicistronic construct harboring FMDV IRES with the primers FMDV IRES F and FMDV IRES R (shown in table 2.2), and inserted in *Bam*HI/*Eco*RI site of pCDNA 3.1(+) vector. All the reporter constructs were confirmed by sequence analysis.

Table 2.1: Sequences of Viral and Cellular IRES elements used in this study.

Table 2.1

Viral and Cellular IRES	Abbreviation	Accession number	Nucleotides
Encephalomyocarditis virus	EMCV	NC_001479	335-839
Foot and mouth disease virus	FMDV	NC_004004	587-1044
Hepatitis C virus	HCV	AB016785	40-407
Human rhinovirus	HRV	NC_001617	1-621
Poliovirus	PV	V01148	109-743
Aquaporin 4	AQP-4	pBICAQP4 plasmid	4578-4869
Apoptotic protease-activating factor1	Apaf-1	NM_013229	1-580
Immunoglobulin heavy chain binding protein	BIP	NM_005347	35-256
Cationic amino acid (arginine/lysine) transporter 1	CAT-1	AF467068	1-273
c-myc proto-oncogene	c-myc	NM_002467	176-527
NF-kappaB repressing factor	NRF	AJ011812	1-652
Cold stress induced mRNA	Rbm3	AY052560	1-720
Vascular endothelial growth factor (VEGF) and type 1 collagen inducible protein	VCIP	NM_003713	1-570

Figure 2.1: Schematic representation of the strategy used to clone the different bicistronic reporter constructs.



5' A cloning site: EMCV, FMDV, HRV, HCV and PV IRES
5' B cloning site: Apaf-1, AQP-4, BIP, CAT-1, c-myc, NRF, Rbm3 and VCIP IRES
3' A cloning site: Apaf-1, AQP-4, BIP, CAT-1, c-myc, NRF, VCIP and HCV IRES
3' B cloning site: EMCV, FMDV, HRV, PV and Rbm3 IRES

Figure 2.1

Table 2.2: Sequences of primers used in this study. Specific primers for Renilla luciferase and firefly luciferase used for qRT-PCR were previously described (Holcik *et al.*, 2005), as well as the primer P2R used for RT-PCR (Van Eden *et al.*, 2004).

Table 2.2

Method	Primer sequence
Cloning	
EMCV F	5'-gccagctgggtatttccaccatattgc-3'
EMCV R	5'-gcccattggggccatattatcatcgtg-3'
FMDV F	5'-gccagctgcacgacgatctaagcaggtttcc-3'
FMDV R	5'-gcccattggagggtcattaattgtaaagg-3'
HCV F	5'-gccaggctcctcccctgtgaggaactact-3'
HCV R	5'-gcgaattcctaactgtcctgtgggcggcgg-3'
PV F	5'-gccagctggacgcacaaaaccaagttaatag-3'
PV R	5'-gctcatgacattatgatacaattgtctg-3'
AQP-4/NRF F (β gal)	5'-cggaattcgacaaactcggcctc-3'
AQP-4/NRF R (CAT)	5'-atgcatggatatccagtatt-3'
FMDV IRES F	5'-cgggatcccacgatctaagcag- 3'
FMDV IRES R	5'-cggaattcagggtcattaattg- 3'
RT-PCR	
Firefly Luciferase F	5'-tcaaagaggcgaactgtgtg-3'
Firefly Luciferase R	5'-tttccgcatcgtctttcc-3'
Renilla Luciferase F	5'-aacgcggcctcttcttattt-3'
Renilla Luciferase R	5'-ttgcctgattgcccata-3'
GAPDH F	5'-gggtggagccaaacgggtca-3'
GAPDH R	5'-ggaggtgctgttgaagtcgca-3'
P1F	5'-ggagaccaatagaaactgggcttgc-3'
P2R	5'-tctcttcatagccttatgcagttgc-3'

Table 2.2 continued

Method	Primer sequence
qRT-PCR	
Firefly Luciferase F	5'- gaggttccatctgcaggta -3'
Firefly Luciferase R	5'- ccggtatccagatccacaac -3'
Renilla Luciferase F	5'- aacgcggcctcttcttattt -3'
Renilla Luciferase R	5'- ttgcctgattgcccata -3'
GAPDH F	5'- atcttcttttgcgtcgccag -3'
GAPDH R	5'- acgaccaaatccgttgactcc -3'

2.2 Cell culture

Murine fibroblast cell line (NIH3T3), human colorectal carcinoma cell line (HT116), human hepatoma cell line (Huh7), human lung fibroblast cell line (MRC5) and monkey kidney epithelium cell line (Vero) were obtained from the American Type Culture Collection (ATCC). Mouse embryonic fibroblasts (MEF) used in Chapter 3 were kindly provided by Dr. Patrick Lee (Dalhousie University, Halifax, Canada). MEF derived from wild type and GCN2 knockout mice used in Chapter 4, were kindly provided by Dr. Nahum Sonenberg (McGill University, Montreal, Canada). All cell lines used in this study were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Ontario, Canada), supplemented with 10% fetal bovine serum (FBS) (Cansera, Etobicoke, Ontario, Canada).

2.3 Reagents

Antibodies against phospho-eIF2B ϵ and Ebp1 were obtained from Upstate, total GCN2, total 4E-BP1 and phospho-4E-BP1 (Thr70) from Cell Signaling Technology, total ERK and hnRNP1 (N20) (PTB) from Santa Cruz Biotechnology. The inhibitor for phosphoinositide-3 kinase (PI3K) (LY294002) was obtained from Calbiochem and Torin1 was kindly provided by Dr. David Sabatini, Whitehead Institute for Biomedical Research (Cambridge, MA).

2.4 DNA Transfection

Cells were plated in 24 well plates and incubated overnight. Bicistronic reporter constructs or control construct (pRF) (1 µg/well) were transfected into cells using Superfect (QIAGEN, Valencia, CA) with the exception of MRC5 cells that were transfected using Polyfect (QIAGEN), according to the manufacturer's instructions.

2.5 Hydrodynamic gene delivery

In vivo gene transfection was performed by hydrodynamic gene delivery (Liu *et al.*, 1999). Six week-old CD1 male mice (22-25g) were injected with 20 µg of bicistronic reporter construct in saline equivalent to 8% mouse body weight by tail vein injection in 5-7 seconds. Twenty-four hours after injection, mice were sacrificed and perfused with PBS. Liver samples were removed, snap frozen and ground to a powder. For luciferase assay, the liver tissue powder was weighed and resuspended in 2X volumes of Passive Lysis Buffer (Promega). The lysates were then centrifuged 10 min at 13,000 g at 4°C and the supernatant assayed for luciferase activity. For measurement of plasmid DNA and plasmid mRNA, liver tissue powder (100mg) was lysed in 1 ml of TRIzol (Invitrogen).

2.6 Luciferase assay

Cells were washed with PBS and lysed with Passive Lysis Buffer (Promega). *Renilla* luciferase and firefly luciferase activities were measure using the Dual-Luciferase Reporter Assay System (Promega). Cell lysates or liver extracts (10 µl) were mixed with

luciferase assay reagent (50 μ l) and luciferase activity was measured as relative light units (RLU) in a Fluoroskan Ascent (Labsystems) luminometer for 10 s.

2.7 RT-PCR and quantitative RT-PCR

RNA was isolated from cell using TRIzol (Invitrogen) according to the manufacturer's instructions. To remove plasmid DNA, isolated RNA was treated with Turbo DNA-free DNase (Ambion, Austin, TX) according to the manufacturer's instructions. For reverse transcriptase polymerase chain reaction (RT-PCR), plasmid DNA free RNA (0.5 μ g) was reverse transcribed (RT) to cDNA from random hexamers using the first-strand cDNA synthesis kit from Amersham Biosciences (Piscataway, NJ). RT-PCR and quantitative RT-PCR (qRT-PCR) were performed using the primers shown in Table 2.2. For qRT-PCR, primers were validated using a 5-point, 5-fold dilution series of pRF plasmid spiked into RNA isolated from untransfected NIH3T3 cells using Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) on the 7500Fast qPCR system (Applied Biosystems, Foster City, CA). The absence of non-specific amplification was confirmed by observing a single peak in the melt-curve analysis, confirmation of the expected amplicon size by agarose gel analysis and the absence of amplification in the no template control wells. For analysis of mRNA transcribed from transfected plasmid, RNA was treated with Turbo DNA-free (Ambion) and cDNA prepared as described above. Quantitative polymerase chain reaction (qPCR) was then performed in triplicate on the StepOnePlus (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Cycling

conditions were: 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min followed by melt-curve analysis.

Polymerase chain reaction (PCR) was performed in the absence of reverse transcriptase on 0.5% of the TRIzol extract containing RNA and plasmid DNA, and copy number calculated by comparison to a 6-point standard curve consisting of a 5-fold dilution series starting at 3×10^4 copies of pRF plasmid spiked into control, untransfected RNA using the conditions described above for primer validation.

2.8 Cellular stress induction

At 24 hours after transfection with bicistronic reporter constructs or control (pRF) construct, cells were exposed to different cellular stresses. For amino acid starvation, cells were incubated with Krebs-Ringer Bicarbonate Buffer (Sigma) with 10% of dialyzed FBS (Invitrogen) for 6 hours. For glucose starvation, cells were incubated with glucose free DMEM (Invitrogen) with 10% dialyzed FBS for 8 hours. For heat shock, cells were incubated at 42°C for 8 hours. For serum starvation, cells were incubated with DMEM with 0.5% FBS for 24 hours. For oxidative stress, cells were treated with 8 μ M of H₂O₂ (Sigma) for 8 hours. For UV irradiation, cells were exposed to 80 J/m² UV-C in the Stratalinker UV crosslinker (Stratagene). For leucine starvation, cells were incubated with L-glutamine, leucine free DMEM (USBiological) with the addition of L-glutamine (Invitrogen). For glutamine starvation, cells were incubated with L-glutamine free DMEM (Invitrogen).

2.9 Western Blot analysis

Cells were washed with PBS and lysed with RIPA buffer containing 0.1 % SDS, 10 µg/ml of aprotinin, 100 µg/ml of phenylmethylsulfonyl fluoride (PMSF) and 1% phosphatase inhibitor cocktail (Sigma). The samples were subjected to SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad). The membrane was blocked with 5% skim milk in TBS containing 0.1% Tween 20 and then incubated with the primary antibody (phospho-eIF2Bε, total ERK, total GCN2, total 4E-BP1, phospho-4E-BP1, hnRNP I or Ebp1) followed by secondary antibody (peroxidase-conjugated anti-rabbit IgG) (Santa Cruz Biotechnology). Specific bands were detected using ECL (Amersham).

2.10 siRNA transfection

NIH3T3 cells were treated with 50 pmol/ml of eIF4E-BP1 ON-TARGETplus SMARTpool small interfering RNAs (siRNA) or scrambled control siRNA (Dharmacon) using DharmaFect1 transfection reagent (Dharmacon) according to the manufacturer's protocol. Forty-eight hours after siRNA transfection, cells were transfected with bicistronic reporter constructs and then subjected to amino acid starvation as described above.

2.11 Protein extract for RNA affinity chromatography

NIH3T3 cells incubated in control or amino acid starved (Krebs Ringer Buffer + 10% dialyzed FBS) conditions were washed twice in PBS, trypsinized, pooled and collected by

centrifugation. The cell pellet was washed twice in ice-cold PBS, and then resuspended in homogenization buffer (10 mM Tris-Cl, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.1 mM PMSF, and 10 µg/ml leupeptin). Cells were lysed with 20-30 strokes with a Dounce Homogenizer on ice. The nuclei were pelleted by centrifugation at 2000g for 10 min (4°C). The supernatant was transferred to a new tube and the KCl concentration was adjusted to 150 mM.

2.12 RNA affinity chromatography

RNA affinity chromatography was performed using a modified protocol (Lewis *et al.*, 2007). Briefly, FMDV IRES RNA (457 bp) cloned in the pcDNA 3.1 vector, was *in vitro* transcribed with RiboMAX Large Scale RNA Production Systems (Promega, Madison, WI), and subsequently polyadenylated with the Poly (A) tailing kit (Ambion, Austin, TX). Polyadenylated transcripts (2.5 µg) were incubated with oligo-dT dynabeads (Invitrogen) in binding buffer (10 mM Tris-Cl, pH 7.4, 150 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM PMSF, 0.05% [vol/vol] Nonidet P-40) at 4°C for 30 minutes on a rotating wheel. Unbound RNA was removed and the beads-RNA complexes were washed twice with the binding buffer. Protein extracts from control or amino acid starved NIH3T3 (100 µg) were added to the beads-RNA complexes in the presence of 120 µg yeast tRNA (Sigma) and 800U of RNase inhibitor (Invitrogen). Reactions were incubated at 4°C with continuous rotation for 1 hour. Unbound proteins were removed by washing with the binding buffer and beads resuspended in 40 µl of 10 mM Tris-HCl + PMSF and incubated at 75°C for 2 minutes. Loading dye 3X SDS-PAGE (20 µl) was added and

samples were boiled for 5 minutes to elute bound proteins. Proteins were separated by 8% SDS-PAGE and subjected to silver staining or western blot analysis.

2.13 Silver staining

SDS-page gels were fixed in fixing solution (ethanol:glacial acetic acid:H₂O, 40:10:50) for 1 hour at room temperature. Gels were rinsed twice with 30% ethanol and then in H₂O, for 20 minutes each wash at room temperature. Gels were sensitized in 0.02% of sodium thiosulfate for 1 minute and rinsed three times in H₂O. Gels were then stained with cold 0.1% silver nitrate solution for 20 minutes at 4°C, rinsed three times in H₂O and then incubated with developing solution (3% potassium carbonate, 0.05% formalin) until the desired contrast was obtained. The reaction was quenched by washing the gel in 1% acetic acid for a few minutes, after which the gel was washed with H₂O.

2.14 Statistical Analysis

One-way ANOVA test with Dunnet's post-test and t-test were used for statistical analysis (GraphPad Prism, GraphPad Software, San Diego, CA).

CHAPTER 3

COMPARISON OF VIRAL AND CELLULAR IRES-MEDIATED TRANSLATION EFFICIENCY IN PHYSIOLOGICAL CONDITIONS

3.1 Rationale

The primary sequences and secondary structures of IRES elements are diverse and similarities have not yet been found among cellular IRES elements or between different viral groups. ITAFs, which interact with IRES elements to regulate translation efficiency by modulating ribosomal recruitment or by modifying IRES structures, also vary with different viral and cellular IRES elements. Because of these diversities, different IRES elements may demonstrate distinct translation efficiencies depending on cell type and cellular conditions. To test this, I constructed bicistronic reporter constructs containing one of 13 different IRES elements and compared their activities *in vitro* and *in vivo* under physiological conditions.

The most commonly used IRES element for current pharmaceutical and biomedical application originates from EMCV. However, it has been reported that expression levels of target genes under control of the EMCV IRES are often insufficient (Li *et al.*, 2007; Mizuguchi *et al.*, 2000; Wang *et al.*, 2005b; Wong *et al.*, 2002) and may not be suitable to achieve therapeutic efficacy in gene therapy. Therefore, the identification of an IRES element(s) with higher translation efficiency under physiological conditions than EMCV IRES element may improve outcomes of applications of bicistronic vectors for experimental and therapeutic purposes.

3.2 Results

3.2.1 Comparison of IRES elements efficiency to initiate protein translation *in vitro*

We first conducted *in vitro* screening with different cell lines representing various cell types of human and mouse origins: NIH3T3, MEF, Huh7 and MRC5. Cells were transfected with a bicistronic pRF construct containing one of the 13 IRES elements or the empty control construct. In this system, a viral promoter directs the synthesis of a single mRNA strand containing an IRES element between open reading frames. The first gene (*Renilla* luciferase) is translated by a cap-dependent, ribosome scanning mechanism with its own 5'-UTR, while translation of the subsequent gene (firefly luciferase) is accomplished by the direct recruitment of ribosomes to the IRES in a cap-independent manner (Figure 3.1). As shown in Figure 3.2A-D, several IRESs initiated significantly higher expression of firefly luciferase compared to EMCV IRES ($p < 0.01$): VCIP IRES in NIH3T3, AQP-4, Rbm3 and VCIP IRES in MEF, c-myc, Rbm3 and VCIP IRES in Huh7, and c-myc and VCIP IRES in MRC5. These results confirmed those from a previous study showing that c-myc IRES initiated protein expression better than EMCV IRES in some cell types (Wong *et al.*, 2002). Importantly, VCIP IRES was the only IRES that demonstrated consistently higher activity relative to EMCV IRES in all the cell lines investigated. Firefly luciferase expression initiated by VCIP IRES was 48-fold higher in NIH3T3, 61-fold higher in MEF, 538-fold higher in Huh7 and 238-fold higher in MRC5 compared to the EMCV IRES.

The presence of an IRES element in bicistronic vectors may interfere with cap-dependent translation of the upstream *Renilla* luciferase gene, which is an undesirable

Figure 3.1: Schematic representation of reporter gene analysis. After cellular transfection, bicistronic reporter construct is transcribed in the nucleus into a bicistronic mRNA molecule. In this system, *Renilla* luciferase is translated by cap-dependent mechanism and firefly luciferase is translated by IRES-mediated mechanism. Translation efficiency of each mechanism is evaluated using dual luciferase assay.

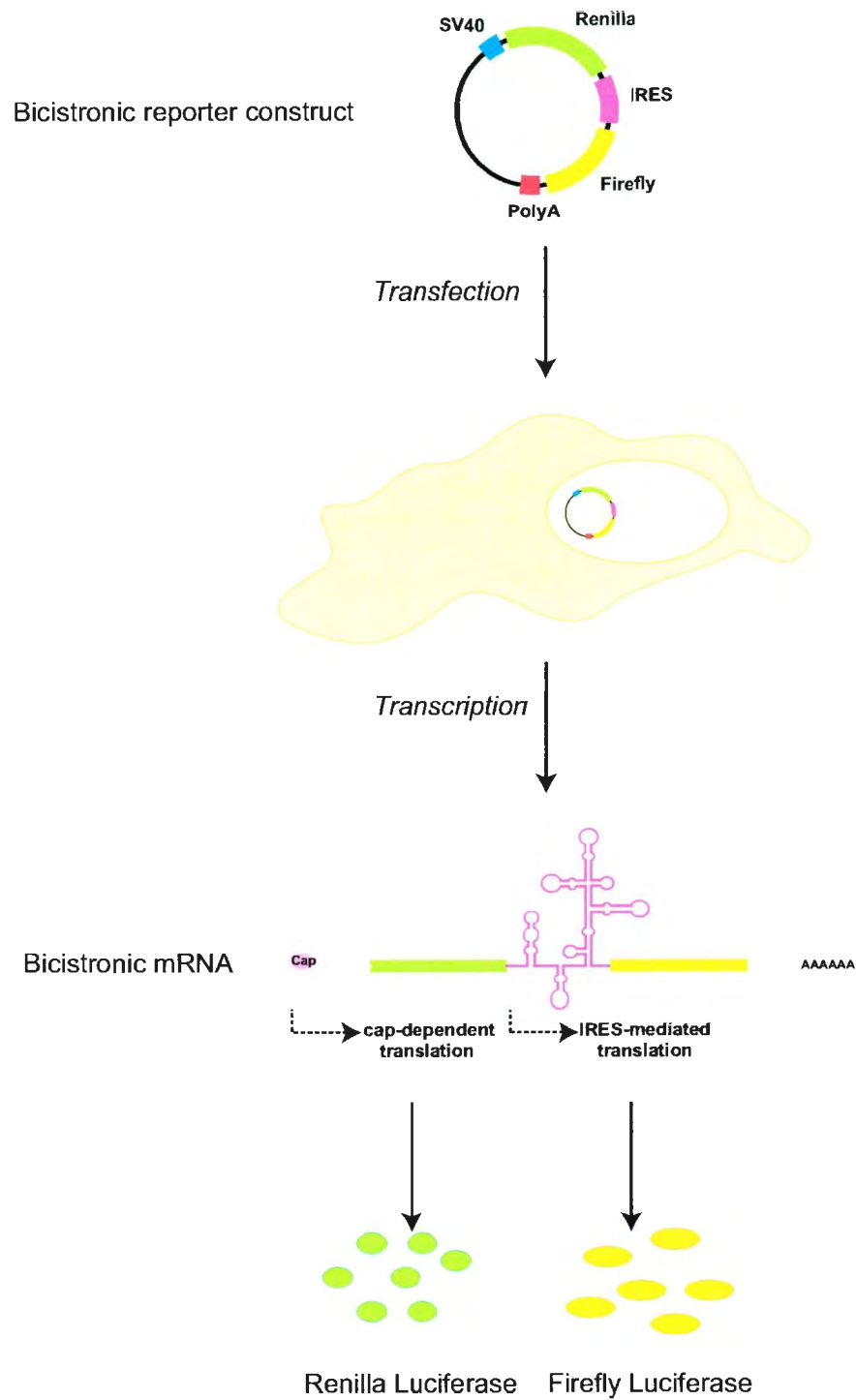


Figure 3.1

Figure 3.2: *In vitro* comparison of IRES elements for their ability to initiate translation. Bicistronic pRF construct containing one of the 13 IRES elements or the control construct (PRF) was transfected into cells. Twenty-four hours after the transfection, firefly (A-D) and *Renilla* (E-H) luciferase activities were measured. Data are mean \pm SE of 3 independent experiments. Statistical analyses were performed with one-way ANOVA test and Dunnet's post-test using EMCV as the reference group. ** $p < 0.01$.

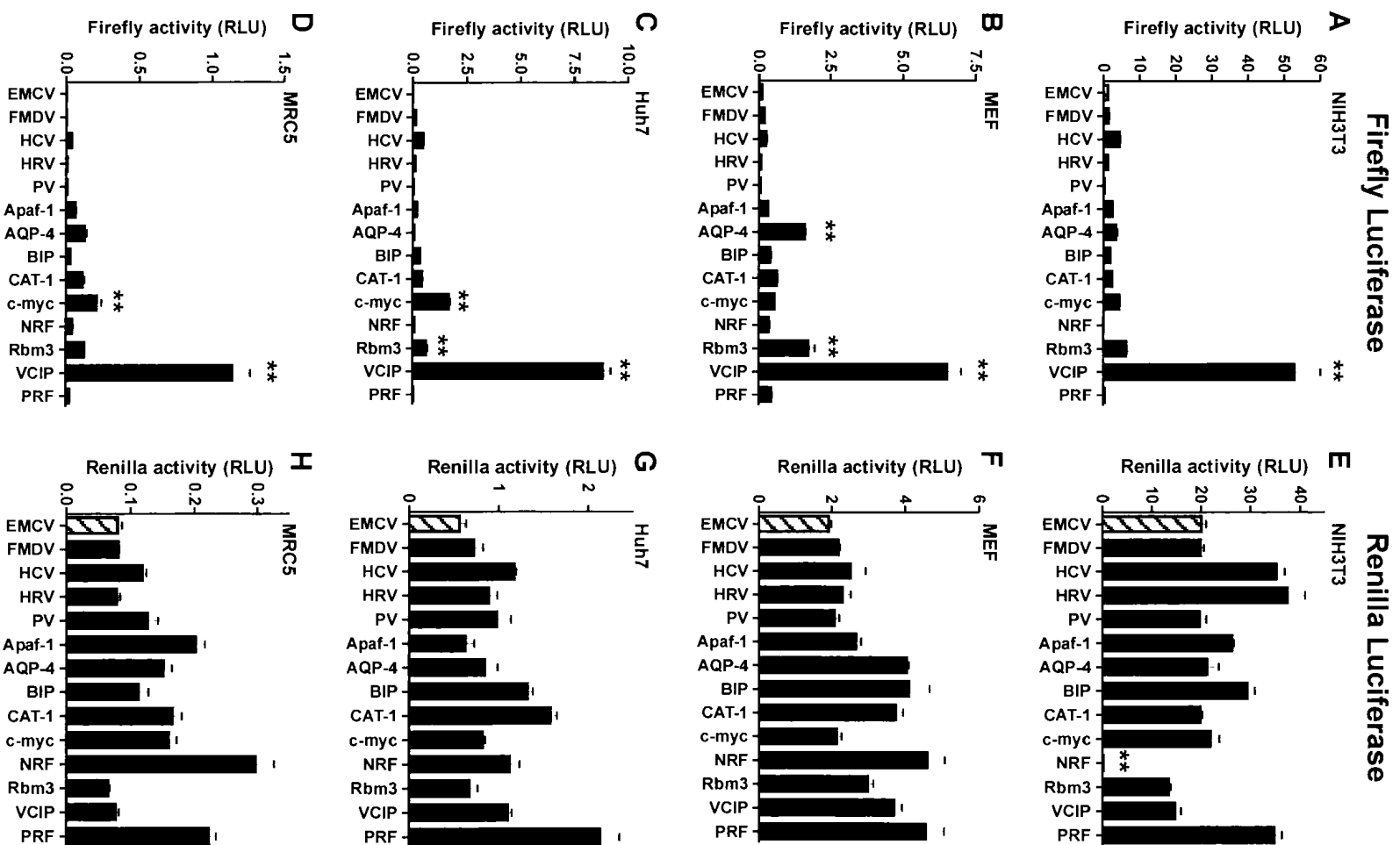


Figure 3.2

outcome for expression vectors. When we compared the expression of *Renilla* luciferase expression (Figure 3.2, right panels), none of the 13 IRES elements showed decreased levels compared to EMCV IRES with the exception of the construct containing NRF IRES element in NIH3T3 cell line (Figure 3.2E). These results demonstrate that there are IRES elements capable of mediating downstream gene expression significantly better than the EMCV IRES without inhibiting cap-mediated expression of the upstream gene in cell culture systems.

3.2.2 Comparison of IRES elements efficiency to initiate protein translation *in vivo*

We next investigated whether the IRES elements that initiated highly efficient protein expression *in vitro* would also exhibit higher efficiencies *in vivo*. Bicistronic constructs with EMCV, HRV, AQP-4, CAT-1, c-myc or VCIP IRES, or a control construct (pRF) were transfected into the liver of mice using hydrodynamic gene delivery (Figure 3.3). This technique involves the injection of a large volume of DNA solution into the blood vessel that permeabilize the endothelial and parenchymal cells allowing DNA entrance due to the hydrodynamic force (Kobayashi *et al.*, 2004; Zhang *et al.*, 2004). The protein expression levels of firefly and *Renilla* luciferase were standardized to the amount of bicistronic construct taken up by the liver (Figure 3.4A and B). We found that the expression of firefly luciferase was 23-fold higher in the livers of mice transfected with the VCIP IRES construct compared to those with the EMCV IRES construct ($p < 0.01$) (Figure 3.4A). In contrast, no significant differences in firefly activity were observed between the other IRES elements (HRV, AQP-4, CAT-1 and c-myc) and EMCV IRES.

Figure 3.3: Schematic representation of Hydrodynamic gene delivery used to evaluate IRES-mediated translation initiation efficiency *in vivo*.

Hydrodynamic gene delivery



Figure 3.3

Figure 3.4: *In vivo* comparison of IRES elements for their ability to initiate translation. Luciferase activities in the livers were normalized either to the amount of bicistronic plasmid DNA (**A** and **B**) or to the amount of bicistronic mRNA (**C** and **D**). Six week-old CD1 male mice (22-25g) were injected with 20 µg of bicistronic reporter construct by hydrodynamic gene delivery. To measure the amount of transfected bicistronic plasmid DNA, quantitative PCR (qPCR) was performed in the absence of reverse transcriptase, and copy number of the plasmid DNA calculated by comparison to a 6-point standard curve consisting of a 5-fold dilution series starting at 3×10^4 copies of control pRF plasmid. To measure the amount of transcribed bicistronic mRNA, firefly and *Renilla* activity (RLU: Relative Light Units) were normalized to the amount of transfected bicistronic plasmid DNA (fg) or that of bicistronic mRNA (copy number) in the liver. Each dot represents one animal (5 animals/group). Data are the median of the ratio of RLU to bicistronic plasmid DNA or bicistronic mRNA. Statistical analyses were performed with one-way ANOVA test and Dunnet's post-test using EMCV as the reference group. ** $p < 0.01$.

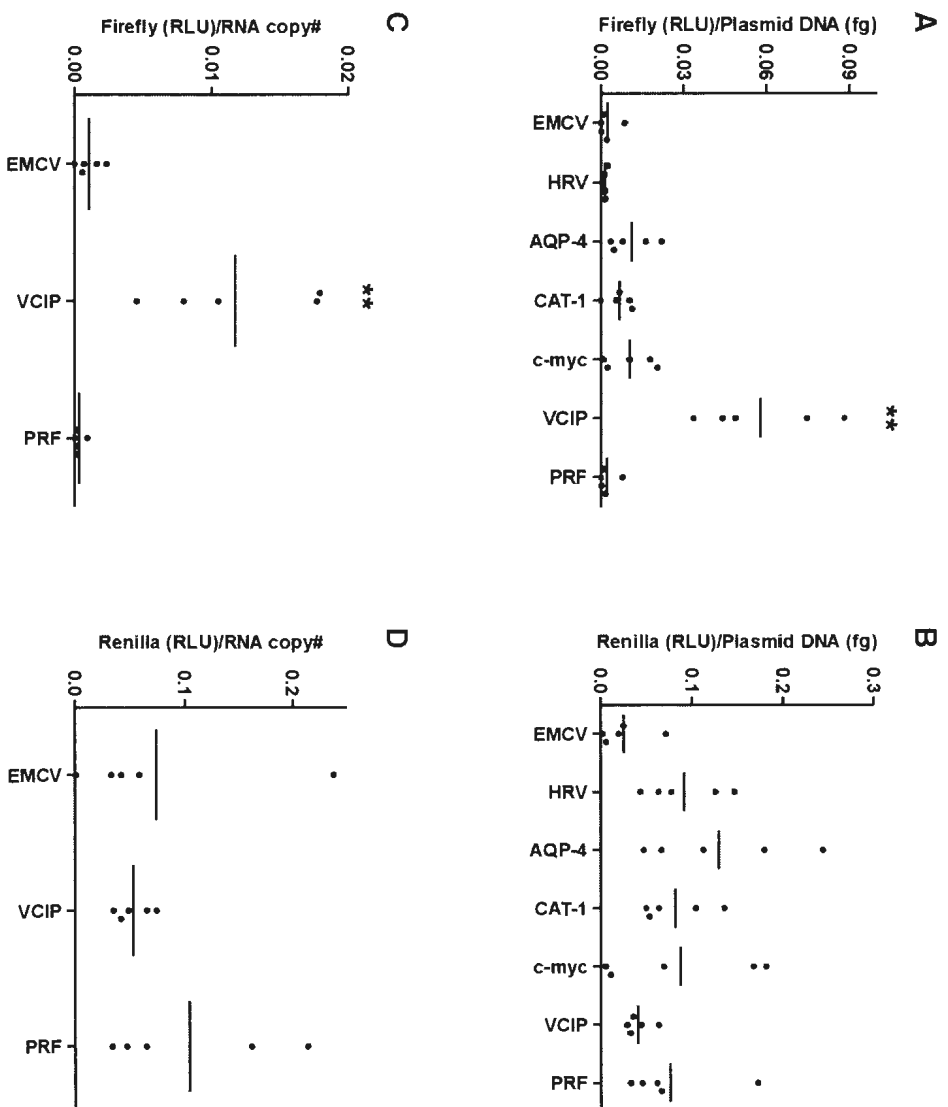


Figure 3.4

Renilla luciferase expression was not significantly different among any of the constructs (Figure 3.4B), suggesting that the presence of IRES in the bicistronic construct does not interfere with cap-dependent translation of the upstream gene *in vivo*. We also confirmed these results by normalizing the luciferase activities in the livers of mice transfected with pRF, EMCV and VCIP constructs to the amount of bicistronic mRNA (Figures 3.4C and D). Overall, these data demonstrate that VCIP IRES, consistent with *in vitro* results, has the ability to initiate IRES-mediated protein expression *in vivo* with a greater efficiency than EMCV IRES, which currently is commonly used in gene therapy and biomedical research.

3.2.3 Influence of IRES element introduction in bicistronic mRNA behavior

The introduction of certain IRES sequences into expression vectors is known to initiate spurious mRNA splicing events (Holcik *et al.*, 2005; Van Eden *et al.*, 2004a). In addition, aberrant transcripts generated from a cryptic promoter present within the putative IRES have been reported (Bert *et al.*, 2006; Kozak, 2003) and the presence of foreign IRES elements in mRNA may also affect the stability of the transcript. Therefore, it is possible that the observed changes in the expression levels of firefly and *Renilla* luciferase protein may be due to regulation at the transcriptional level and not at the translational level. To clarify this, the transcription and stability of bicistronic mRNAs were examined. We first conducted RT-PCR analysis for *Renilla* and firefly luciferase genes on RNA isolated from NIH3T3 cells transfected with the various constructs (Figure 3.5A). Both *Renilla* and firefly luciferase mRNAs were expressed to a similar level from all bicistronic

Figure 3.5: Absence of bicistronic mRNA modification in response to inclusion of IRES sequence. **A)** RT-PCR on *Renilla* and firefly luciferase mRNA, as well as GAPDH mRNA was performed on total RNA isolated from NIH3T3 cells transfected for 24 hours with bicistronic reporter constructs, including omission of Reverse Transcriptase (RT-), positive control (plasmid) and no template control (NTC). **B)** RT-qPCR was performed to quantify *Renilla* and firefly luciferase mRNA. The *Renilla* luciferase/firefly luciferase ratio was calculated as $2^{-[CT (Renilla) - CT (Firefly)]}$. Data are mean \pm SE of 5 independent experiments. Statistical analyses were performed with one-way ANOVA test and Dunnet's post-test using EMCV as the reference group. **p<0.01.

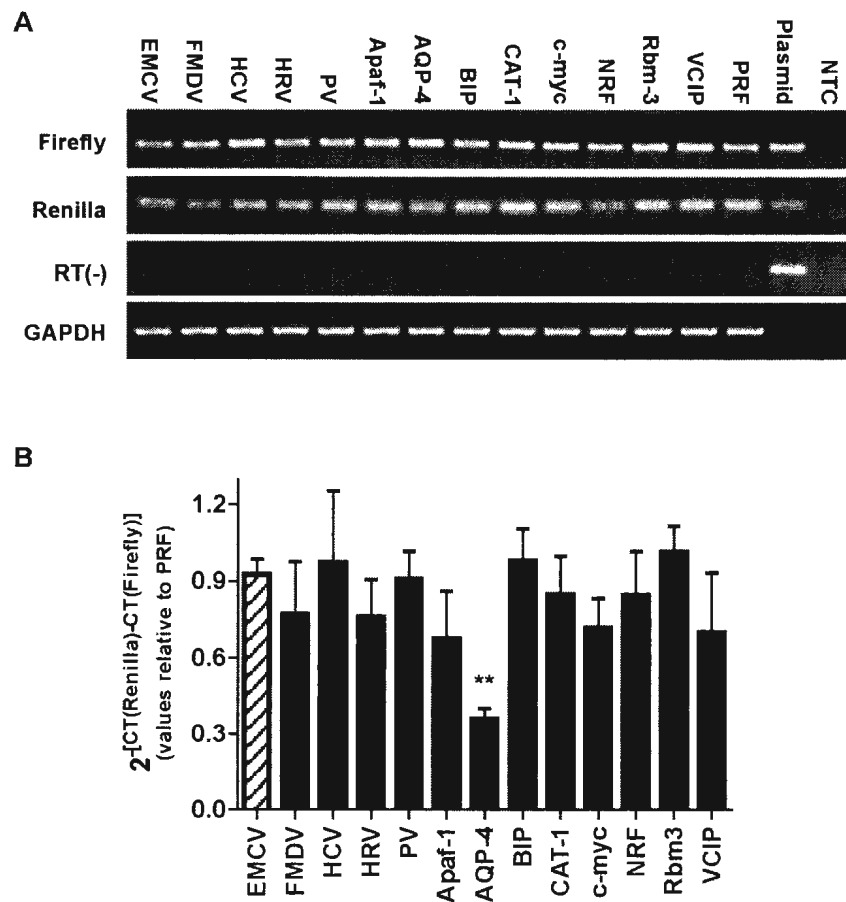


Figure 3.5

constructs tested indicating that the presence of these IRES sequences in the mRNA does not noticeably affect the stability of the transcript. Next, using qRT-PCR we determined whether mRNA splicing or cryptic promoters are generated by the introduction of an IRES element (Figure 3.5B). In the presence of splicing or a cryptic promoter, the ratio of the expression levels of *Renilla* and firefly luciferase mRNA would change. We found significant decreased levels of *Renilla* luciferase mRNA compared with those of firefly luciferase mRNA in AQP-4 construct, whereas we did not observe significant differences in the *Renilla*/firefly ratio in any of the other constructs compared to EMCV IRES construct. We further examined AQP-4, EMCV, VCIP and pRF transcripts by RT-PCR, and found that the AQP-4 construct, but not the other bicistronic constructs, expressed multiple transcripts, suggesting possible presence of splicing (Figure 3.6). Taken together, these results suggest that the introduction of the IRES elements examined in this study does not affect the stability of bicistronic mRNA and, with the exception of AQP-4 IRES, do not create alternative transcripts.

Figure 3.6: Insertion of the AQP-4 IRES element into the bicistronic pRF construct produced multiple splice variants from the bicistronic mRNA. **A)** Schematic representation of the bicistronic reporter construct. The IRES sequence was inserted between the *Renilla* and firefly luciferase genes in the pRF construct. Arrows indicate the positions of P1F and P2R primers that were used for RT-PCR analysis. **B)** RT-PCR was performed on total RNA isolated from transfected NIH3T3 cells with indicated construct. While intact bicistronic mRNA was observed as a single band in cells transfected with EMCV, VCIP and pRF reporter constructs, 4 different sizes of transcripts including the full length transcript and lower amount of three other shorter variants (*) were observed in cells transfected with AQP-4 reporter construct, indicating a possible splicing event.

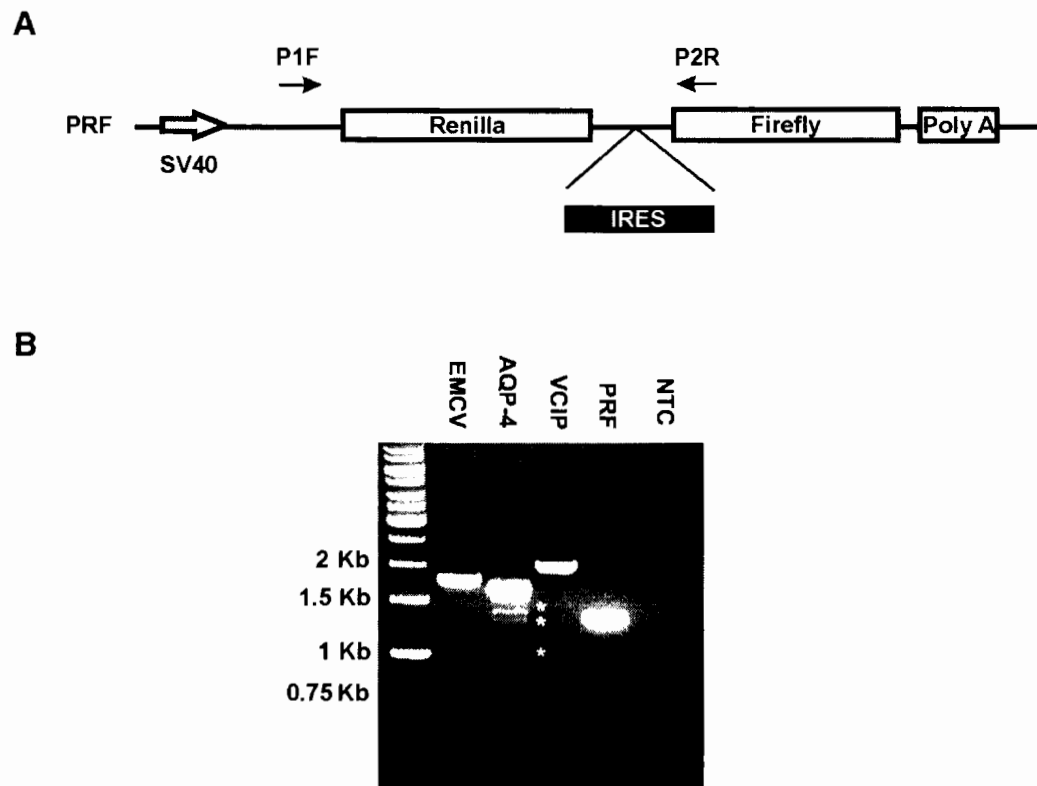


Figure 3.6

CHAPTER 4

**PROMOTION OF VIRAL IRES-MEDIATED TRANSLATION UNDER
CELLULAR STRESS CONDITIONS**

4.1 Rationale

Under cellular stress conditions, cells activate stress response pathways and suppress cap-dependent translation. In contrast, translation mediated by cellular IRES elements often remains active under cellular stress conditions. It is assumed that this is part of a fail-safe strategy in which cellular IRES-mediated translation maintains cellular homeostasis or promotes cell death. For viruses, the translational machinery driven by IRES elements may be a great advantage for their replication, since efficient synthesis of viral proteins can be achieved during cell death or apoptosis.

In contrast to cellular IRES-mediated translation, however, few studies have addressed the efficiency of viral IRES-mediated translation under cellular stresses. In this study, we investigated whether any cellular stresses can promote translation initiation by viral IRES elements.

4.2 Results

4.2.1 Identification of cellular stresses that promote viral IRES-mediated translation

To evaluate the efficiency of viral IRES-mediated and cap-dependent translation under cellular stresses, we used pRF bicistronic reporter constructs containing one of either EMCV, FMDV, HCV, HRV or PV IRES elements. NIH3T3 cells were transfected with the bicistronic reporter construct or the control pRF construct, and 24 hours later subjected to different cellular stresses including amino acid starvation, glucose starvation,

heat shock, oxidative stress, serum starvation or UV irradiation (Figure 4.1). EMCV and FMDV IRES-mediated translation was significantly increased under amino acid starvation, whereas cap-dependent translation was reduced by approximately 50% (Figure 4.1A). As a result, the ratio of EMCV and FMDV IRES-mediated translation to cap-dependent translation was significantly increased under amino acid starvation. In addition, although HRV and PV also gave a higher ratio, this was a result of decreased cap-dependent translation and sustained IRES-mediated translation, but not that of increased IRES-mediated translation. The other cellular stresses we tested did not promote translation mediated by any viral IRES elements nor change the ratio between viral IRES-mediated and cap-dependent translation (Figure 4.1B-F).

4.2.2 Amino acid starvation and viral IRES-dependent translation

To further confirm the regulation of viral IRES-mediated translation under amino acid starvation, we examined whether the efficiency of translation initiation by the FMDV IRES element was enhanced in response to decreasing concentrations of amino acids. NIH3T3 cells transfected with FMDV IRES or control pRF reporter constructs were incubated with culture medium containing different amounts of amino acids (1X, 0.5X, 0.25X, 0.125X of concentration of standard DMEM or no amino acids) for 6 hours. Reduction of amino acid in culture medium increased FMDV IRES-mediated translation in a concentration dependent manner (Figure 4.2A). In addition, the translation ratio between cap-dependent and FMDV IRES-mediated translation was significantly increased. We also determined if the promotion of FMDV IRES mediated-translation by

Figure 4.1: Cellular stresses and viral IRES-mediated translation. NIH3T3 cells were transfected with bicistronic reporter constructs containing one IRES element from EMCV, FMDV, HCV or HRV, or a control pRF construct. 24 hours post-transfection, cells were subjected to different cellular stresses. **A)** Amino acid starvation: cells were incubated in control or amino acid-starved medium for 6 hours. **B)** Glucose starvation: cells were incubated in control or glucose-starved (no glucose DMEM) medium for 12 hours. **C)** Heat shock: cells were incubated at 42°C or in control conditions (37°C) for 8 hours. **D)** Oxidative stress: cells were treated with or without hydrogen peroxide (8 μ M) for 8 hours. **E)** Serum starvation: cells were incubated in control media or serum-starved media for 24 hours. **F)** UV irradiation: the cells were exposed to 80 J/m² of UV-C or left untreated and then lysed at 3 hours after the treatment. Firefly and *Renilla* luciferase units (RLU) were measured using the Dual-Luciferase Reporter Assay System. Firefly/*Renilla* represents the ratio of viral IRES-mediated translation to cap-dependent translation. White bars represent control condition while black bars represent cellular stress condition. Data are mean \pm SE of 3 independent experiments. Statistical analysis was conducted using a t-test. **p<0.01.

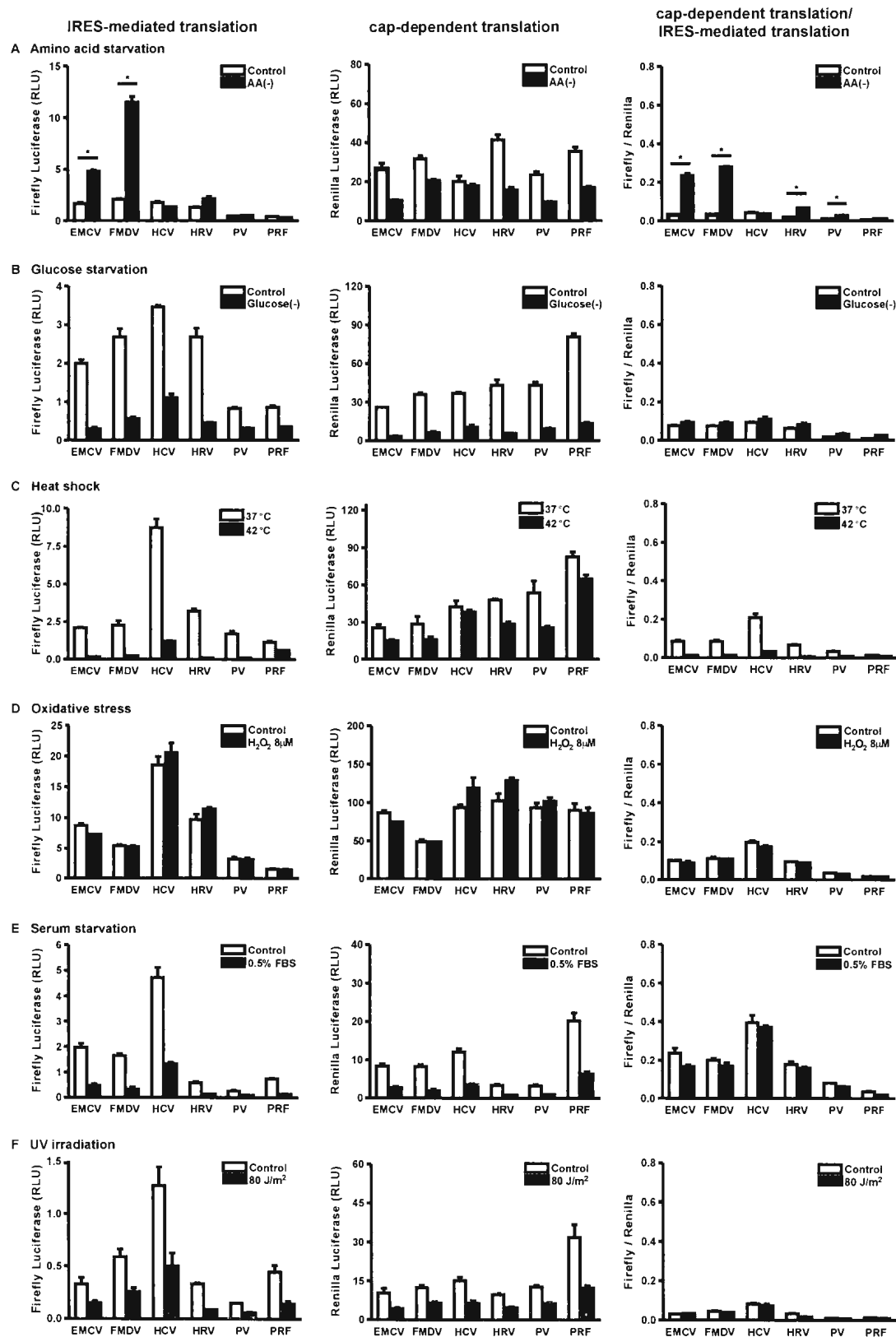


Figure 4.1

Figure 4.2: Amino acid concentration dependent promotion of the viral IRES-mediated translation. NIH3T3 cells were transfected with FMDV IRES (black bar) or control pRF (white bar) reporter constructs. 24 hours later, cells were incubated in medium containing different amino acid concentrations (1X, 0.5X, 0.25X, 0.125X of amino acid concentration of standard DMEM, or no amino acids) for 6 hours. Firefly and *Renilla* luciferase units (RLU) were measure using the Dual-Luciferase Reporter Assay System. Firefly/*Renilla* represents the ratio of viral IRES-mediated translation to cap-dependent translation. Data are mean \pm SE of 3 independent experiments. Statistical analysis was conducted using a t-test. * $p < 0.05$, ** $p < 0.01$.

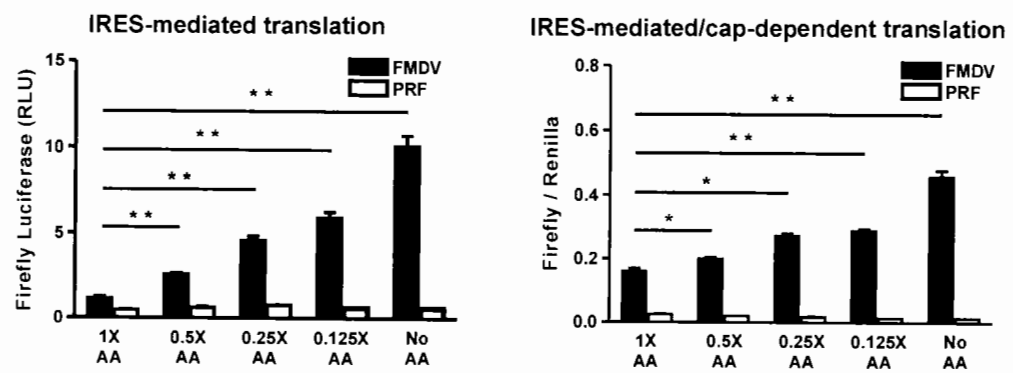


Figure 4.2

amino acid starvation is a cell-type specific phenomenon (Figure 4.3). HCT116, Huh7, NIH3T3 and Vero cell lines were transfected with FMDV IRES or control pRF reporter construct and then subjected to amino acid starvation. We found the promotion of FMDV IRES-mediated translation and the increases in the ratio of FMDV IRES-mediated to cap-dependent translation in HCT116 cells, NIH3T3 cells and Vero cells, but not in Huh7 cells. This suggests that the cellular machinery responsible for the promotion of the IRES-mediated translation is not commonly present in all different types of cell lines.

4.2.3 Amino acid starvation and cellular IRES-dependent translation

Amino acid starvation has been previously shown to increase translation initiation mediated by CAT-1 IRES element in C6 rat glioma cells while cap-dependent translation is severely impaired (Fernandez *et al.*, 2001). To investigate whether the feature of FMDV and EMCV IRES elements can be shared by some cellular IRES elements, NIH3T3 cells were transfected with bicistronic reporter constructs containing one of Apaf-1, BIP, CAT-1 and c-myc IRES elements or the control (pRF) construct for 24 hours and then treated with amino acid starvation for 6 hours (Figure 4.4). Amino acid starvation did not increase translation initiation by any of cellular IRES elements we tested in our experimental system.

Figure 4.3: Cell line-dependent promotion of the viral IRES-mediated translation under amino acid starvation. NIH3T3, HCT116, Huh7 and Vero cells were transfected with FMDV IRES or control pRF reporter construct and 24 hours later, incubated in control (white bar) or amino acid-starved (black bar) medium. Firefly and *Renilla* luciferase units (RLU) were measure using the Dual-Luciferase Reporter Assay System. Firefly/*Renilla* represents the ratio of viral IRES-mediated translation to cap-dependent translation. Data are mean \pm SE of 3 independent experiments. Statistical analysis was conducted using a t-test. * $p < 0.05$, ** $p < 0.01$.

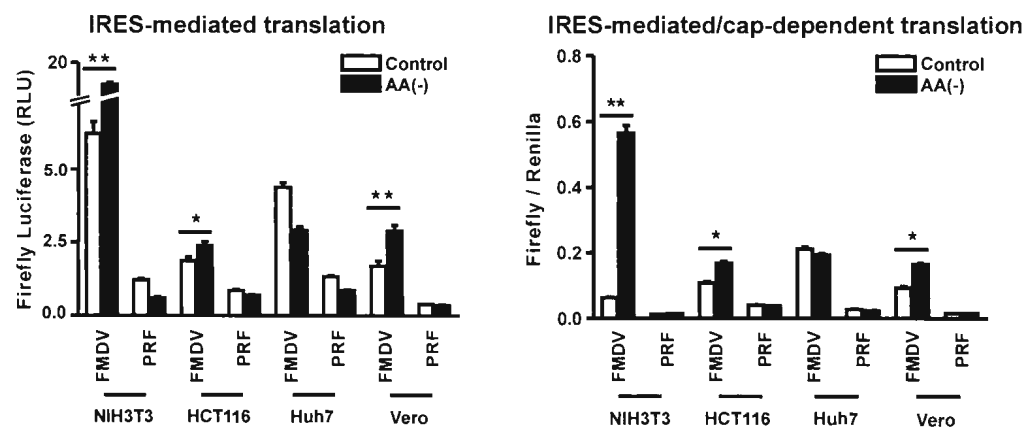


Figure 4.3

Figure 4.4: IRES-mediated translation of cellular IRES elements under amino acid starvation. NIH3T3 cells were transfected with different bicistronic reporter constructs containing cellular IRES, FMDV IRES or control pRF reported constructs and 24 hours later incubated in control (white bars) or amino acid-starved (black bars) medium for 6 hours. Firefly and *Renilla* luciferase units (RLU) were measure using the Dual-Luciferase Reporter Assay System. Firefly/*Renilla* represents the ratio of viral IRES-mediated translation to cap-dependent translation. Data are mean \pm SE of 3 independent experiments. Statistical analysis was conducted using a t-test. * $p < 0.05$, ** $p < 0.01$.

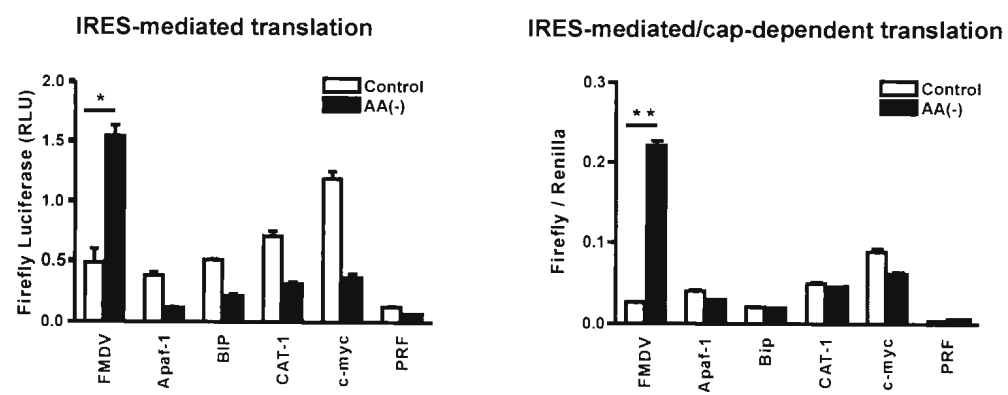


Figure 4.4

4.2.4 Effects of amino acid starvation on transcription of bicistronic mRNAs

It was demonstrated in the previous chapter (Figure 3.3) that the reporter constructs used in this study do not generate spurious mRNA splicing events nor have a cryptic promoter. However, it is still possible that the cellular stress caused by amino acid starvation may modulate transcription and/or integrity of the reporter mRNAs, contributing to the changes in the luciferase activities. To examine this possibility, we compared expression levels of firefly and *Renilla* luciferase reporter mRNA in NIH3T3 cells under amino acid starvation by real time RT-PCR (Figure 4.5). The expression levels of firefly and *Renilla* luciferase mRNA were not affected by amino acid starvation, confirming that the increase of firefly luciferase under amino acid starvation is induced at the translational level, but not at the transcriptional level.

Figure 4.5: Expression of bicistronic reporter mRNA under amino acid starvation.

NIH3T3 cells were transfected with EMCV IRES, FMDV IRES or control pRF reporter construct and 24 hours later, incubated in control (white bar) or amino acid-starved (black bar) medium for 12 hours. **A)** RT-qPCR was performed to quantify *Renilla* and firefly luciferase, and GAPDH mRNA. **B)** The expression ratios of *Renilla* and firefly luciferase mRNA was calculated as $2^{-[CT(Renilla) - CT(firefly)]}$.

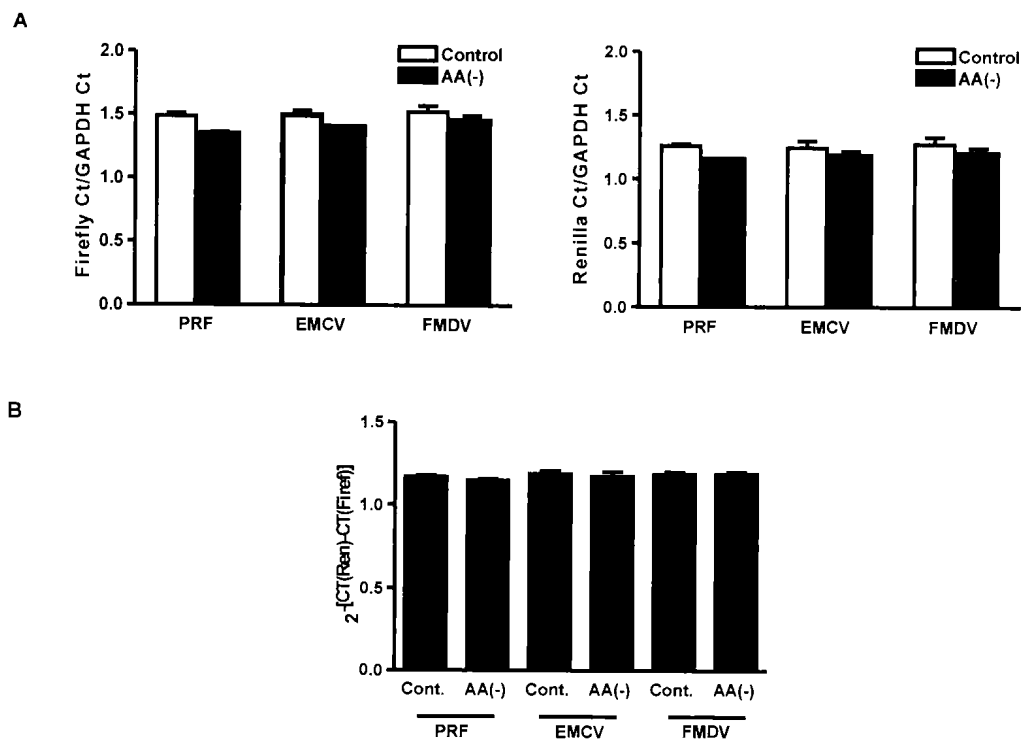


Figure 4.5

CHAPTER 5

CELL SIGNALING INVOLVED IN THE PROMOTION OF FMDV AND EMCV IRES-MEDIATED TRANSLATION UNDER AMINO ACID STARVATION

5.1 Rationale

In Chapter 4, we demonstrated that amino acid starvation promotes FMDV and EMCV IRES-mediated translation while cap-dependent translation is severely impaired. In this chapter, we sought to identify cellular mechanisms responsible for the promotion of EMCV and FMDV IRES-mediated translation under amino acid starvation. Amino acid starvation is known to suppress cap-dependent translation through three different cellular stress response pathways: mTOR-4E-BPs (Kimball, 2001), GCN2-eIF2 α (Harding et al., 2000), and eIF2B (Wang & Proud, 2008) (Figure 1.4). We hypothesized that these pathways might also be involved in the promotion of viral IRES-mediated translation. In addition, it has been suggested that the stress response pathways modulate binding of ITAFs to IRES elements under cellular stress conditions to regulate their translation initiation (Holcik & Sonenberg, 2005; Spriggs et al., 2008). Therefore, we conducted preliminary experiments to determine whether amino acid starvation modulates the binding status of ITAFs to the FMDV IRES element.

5.2 Results

5.2.1 Involvement of the mTOR-4E-BP pathway in the regulation of EMCV and FMDV IRES mediated translation

To determine the involvement of the mTOR-4E-BPs pathway, NIH3T3 cells were treated with a siRNA targeting 4E-BP1 or a scrambled siRNA, or left untreated (Figure 5.1).

Two days after the siRNA treatment, the cells were further transfected with EMCV IRES,

Figure 5.1: Involvement of 4E-BP1 in the promotion of FMDV and EMCV IRES-mediated translation under amino acid starvation. **A)** Knockdown of 4E-BPs by 4E-BP1 siRNA treatment. NIH3T3 cells were transfected with 4E-BP1 siRNA or left untreated (C) for 1 to 4 days. Western blot analysis using antibodies against total 4E-BP1 (t-4E-BP1) and total ERK (t-ERK). **B)** Promotion of viral IRES-mediated translation in 4E-BP1 siRNA knockdown cells under amino acid starvation. NIH3T3 cells were transfected with 4E-BP1 siRNA, non-specific siRNA (Scrambled siRNA) or left untransfected (Control). 48 hours post-siRNA treatment, cells were transfected with EMCV IRES, FMDV IRES or control pRF reporter constructs and 24 hours later incubated in control (white bar) or amino acid-starved (AA(-)) (black bar) medium for 6 hours. Firefly and *Renilla* luciferase units (RLU) were measured using the Dual-Luciferase Reporter Assay System. Firefly/*Renilla* represents the ratio of viral IRES-mediated translation to cap-dependent translation. The bars represent the average \pm SE of 3 independent experiments. Statistical analysis was conducted using a t-test. ** $p < 0.01$.

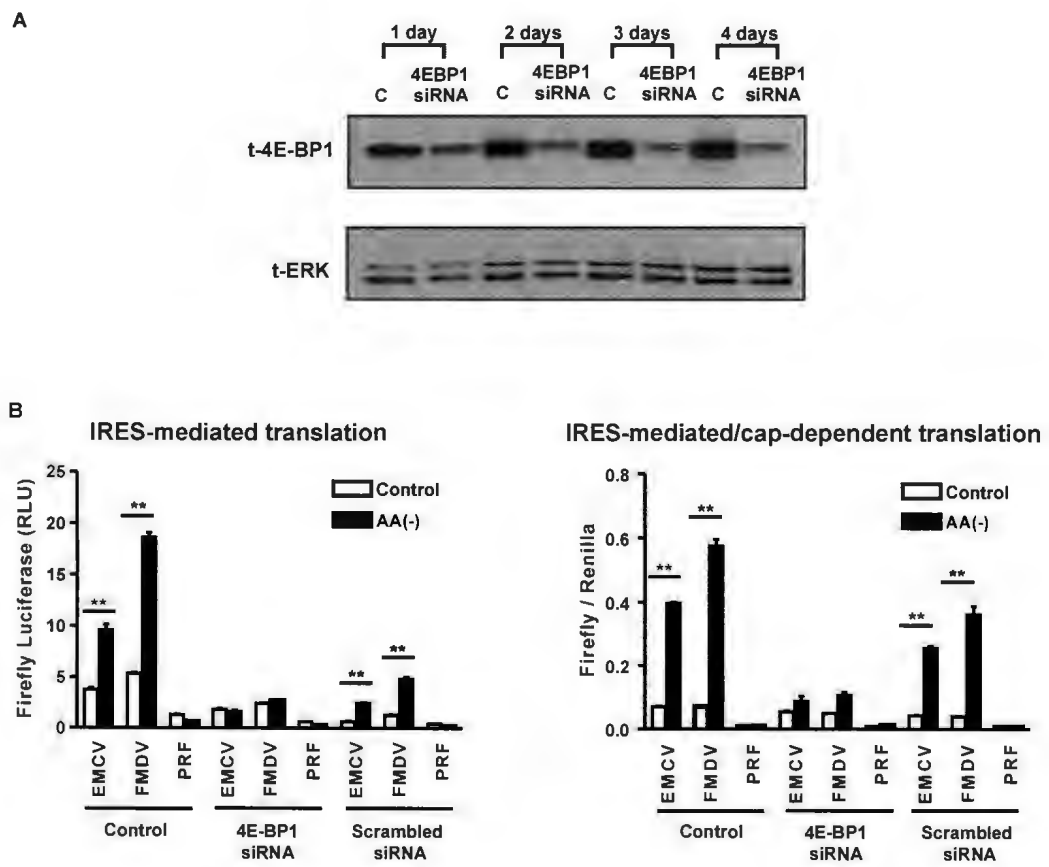


Figure 5.1

FMDV IRES or control pRF reporter construct for 24 hours and then subjected to amino acid starvation for 6 hours. The expression level of 4E-BP1 was effectively reduced in cells treated with the 4E-BP1 siRNA for 4 days (Figure 5.1A). Amino acid starvation significantly promoted EMCV and FMDV IRES-mediated translation in untreated cells and cells transfected with scrambled siRNA, whereas no promotion was observed in cells treated with 4E-BP1 siRNA (Figure 5.1B). Transfection of 4E-BP1 siRNA also abrogated the promotion in the ratio of IRES-mediated and cap-dependent translation (Figure 5.1C). Cells treated with scrambled siRNA showed decreased activity of both firefly and *Renilla* luciferase compared with the untreated cells. Since the ratio of firefly to *Renilla* luciferase was unmodified, this data suggests that this is due to reduced transfection efficiency of EMCV and FMDV bicistronic constructs in siRNA treated cells. These results clearly demonstrate that 4E-BP1 is involved in the promotion of EMCV and FMDV IRES-mediated translation during amino acid starvation.

5.2.2 No involvement of GCN2 and eIF2B ϵ in the promotion of FMDV and EMCV IRES-mediated translation under amino acid starvation

To determine the involvement of the GCN2 pathway, the translation initiated by EMCV and FMDV IRES elements under amino acid starvation were examined in GCN2 knockout MEFs and their wild type counterpart (Figure 5.2). The ratio changes of the viral IRES-mediated and cap-dependent translation under amino acid starvation was not affected by the absence of GCN2, suggesting that GCN2 is not involved in the regulation of the viral IRES-mediated translation. Amino acid starvation has also been shown to

Figure 5.2: No involvement of GCN2 in the promotion of EMCV and FMDV IRES-mediated translation under amino acid starvation. Wild type (WT) and knockout (GCN2^{-/-}) mouse embryonic fibroblasts (MEFs) were transfected with EMCV IRES, FMDV IRES or control pRF reporter constructs and 24 hours later, incubated in control (white bar) or amino acid-starved (black bar) medium for 12 hours. Firefly/Renilla represents the ratio of viral IRES-mediated translation to cap-dependent translation (left and middle). The bars represent the average \pm SE of 3 independent experiments. Statistical analysis was conducted with a t-test. * $p < 0.05$, ** $p < 0.01$. Western blot analysis was performed using antibodies against total GCN2 (t-GCN2) and total ERK (t-ERK) on cell lysates prepared from with wild type (WT) and GCN2 knockout (GCN2^{-/-}) MEF (right).

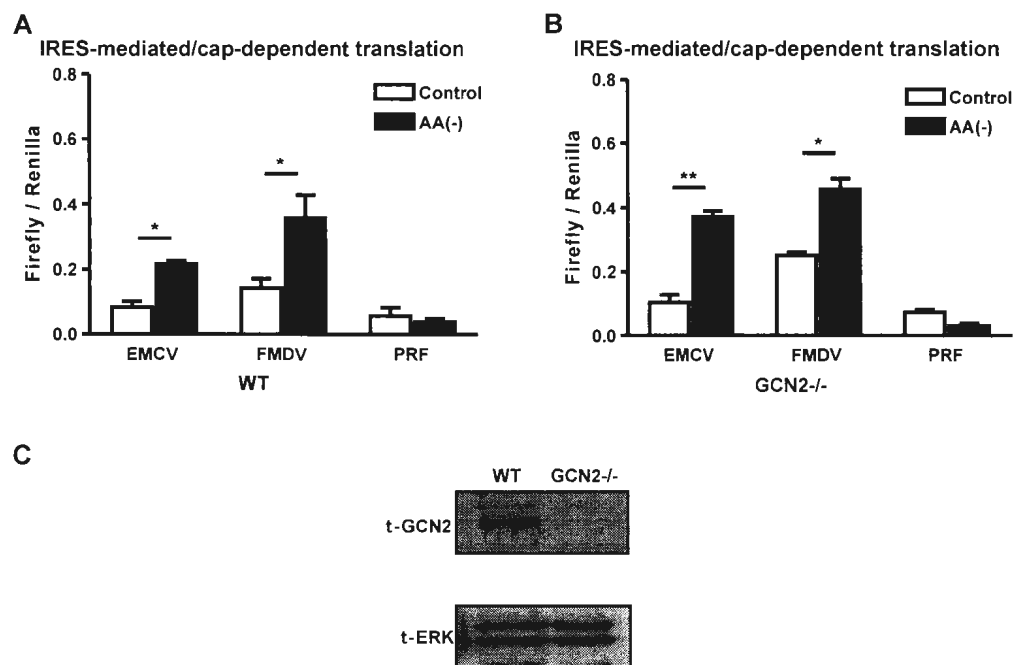


Figure 5.2

Figure 5.3: No involvement of eIF2B ϵ in the promotion of FMDV and EMCV IRES-mediated translation under amino acid starvation. Cell lysates were prepared from NIH3T3 cells incubated in control (DMEM + 10% dialyzed FBS) or amino acid-starved (Krebs-Ringer Bicarbonate Buffer + 10% dialyzed FBS) (AA(-)) medium at the indicated time points. Western blot analysis was performed using antibodies against phosphorylated eIF2B ϵ (p-eIF2B ϵ) and total ERK (t-ERK).

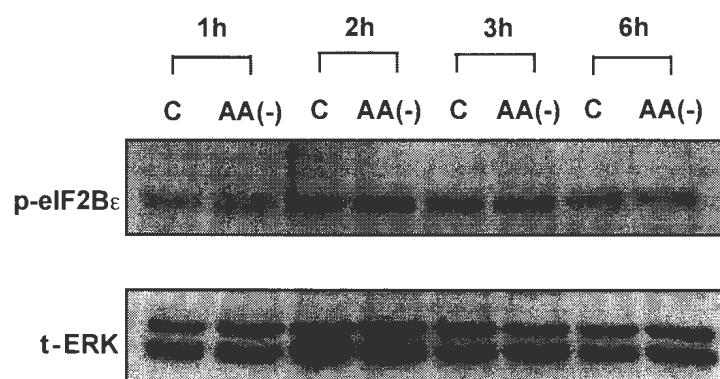


Figure 5.3

directly inhibit eIF2B activity by modulating one of its subunits, eIF2B ϵ , resulting in the suppression of cap-dependent translation (Wang & Proud, 2008). To confirm this, we conducted western blot analysis to examine whether phosphorylation of eIF2B ϵ is modulated by amino acid starvation in our experimental system (Figure 5.3). No changes in eIF2B ϵ phosphorylation were observed in NIH3T3 cells under amino acid starvation compared to control cells. Therefore, it is unlikely that eIF2B plays roles in regulating EMCV and FMDV IRES-mediated translation. Overall, these results demonstrate that the mTOR-4E-BPs pathway is involved in the promotion of translation initiation by EMCV and FMDV IRES elements under amino acid starvation.

5.2.3 Involvement of dephosphorylation of 4E-BP1 in the regulation of EMCV and FMDV IRES-mediated translation

Dephosphorylated 4E-BPs induced by amino acid starvation block the binding of eIF4E to the 5'-cap structure, thereby inhibiting cap-dependent translation. A decrease in active cap-dependent translation may free initiation factors, allowing them to bind the viral IRES elements, resulting in enhanced translation initiation. To test this hypothesis, we determined whether 4E-BP1 dephosphorylation leads to the promotion of EMCV and FMDV IRES-mediated translation. To mimic 4E-BP1 dephosphorylation induced by amino acid starvation, we used LY294002 to inhibit PI3K and Torin1 to inhibit the mTOR pathway, which are upstream pathways of 4E-BPs. Amino acid starvation, or treatment with LY294002 and Torin1, all decreased the phosphorylation level of 4E-BP1 to similar levels (Figure 5.4A). When NIH3T3 cells transfected with EMCV or FMDV

Figure 5.4A: Dephosphorylation of 4E-BP1 induced by LY294002, Torin1 or leucine starvation treatment. Western blot analysis using antibodies targeting phosphorylated 4E-BP1 (p-4E-BP1) and total ERK (t-ERK) on cell lysates prepared from NIH3T3 incubated in control medium (C), amino acid-starved medium (AA(-)), control medium containing LY294002 (LY, 50 μ M), control medium containing Torin1 (T1, 250 nM), Leucine-starved medium (L(-)) or Glutamine-starved medium (G(-)) for the indicated time periods.

A

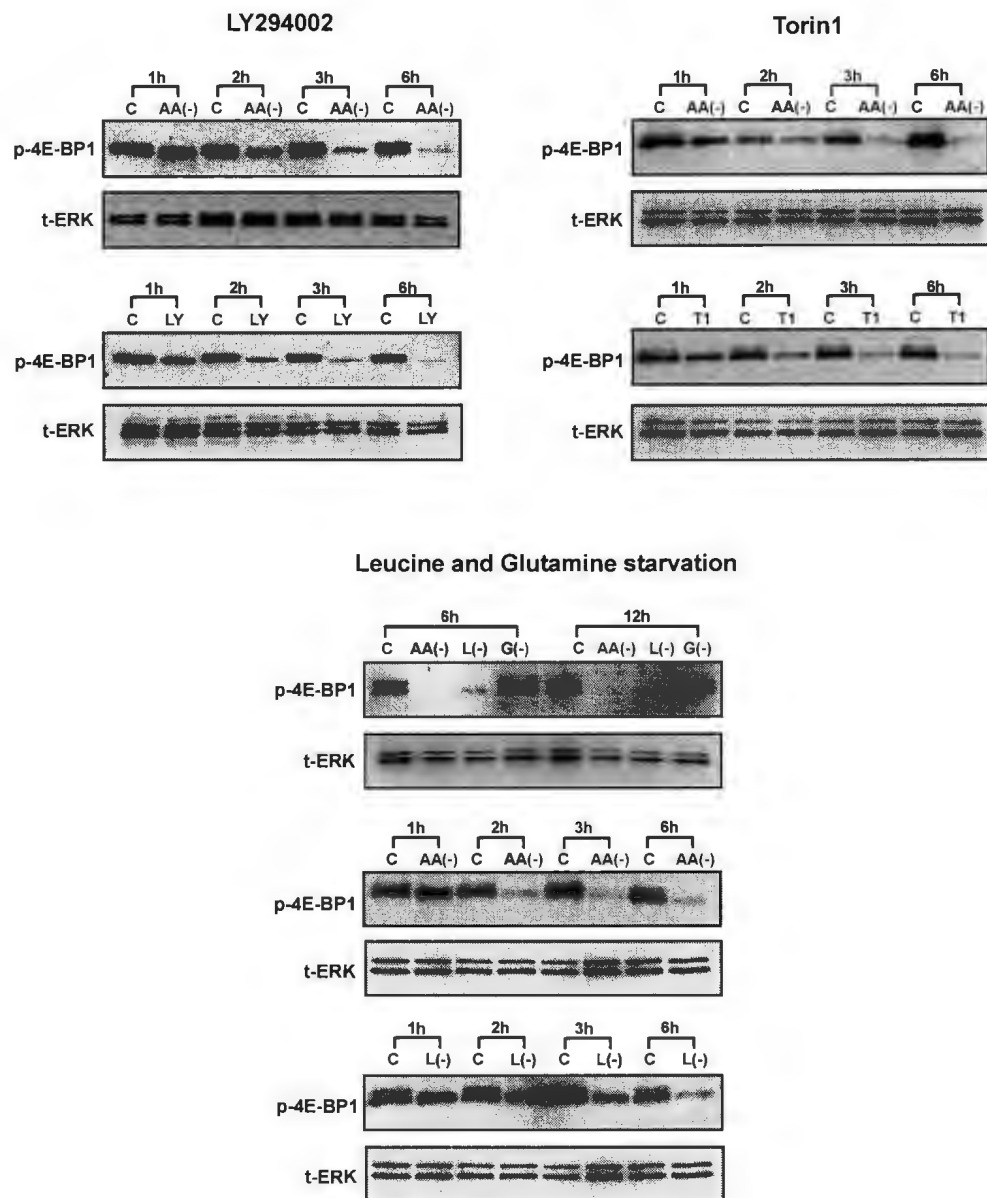
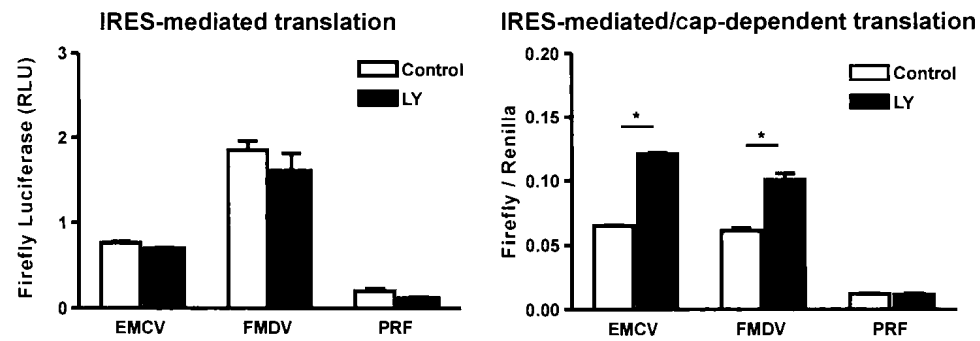


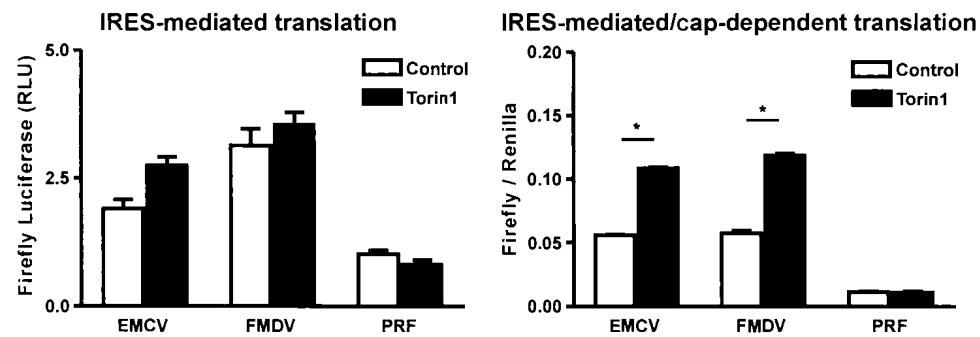
Figure 5.4A

Figure 5.4B-D: EMCV and FMDV IRES-mediated translation under LY294002, Torin1 or leucine starvation treatment. NIH3T3 cells transfected with EMCV IRES, FMDV IRES or control pRF reporter constructs were incubated with or without **(B)** LY294002 (LY, 50 μ M) **(C)** Torin1 (T1, 250 nM), and **(D)** with control, complete amino acid starved (AA (-)), Leucine-starved (L(-)) or Glutamine-starved (G(-)) medium for 6 hours. Firefly and *Renilla* luciferase units (RLU) were measure using the Dual-Luciferase Reporter Assay System. Firefly/*Renilla* represents the ratio of viral IRES-mediated translation to cap-dependent translation. White bars represent control condition while black bars represent cellular stress condition. Data are mean \pm SE of 3 independent experiments. Statistical analysis was conducted using a t-test. * $p < 0.05$, ** $p < 0.01$.

B LY294002



C Torin1



D Leucine and Glutamine starvation

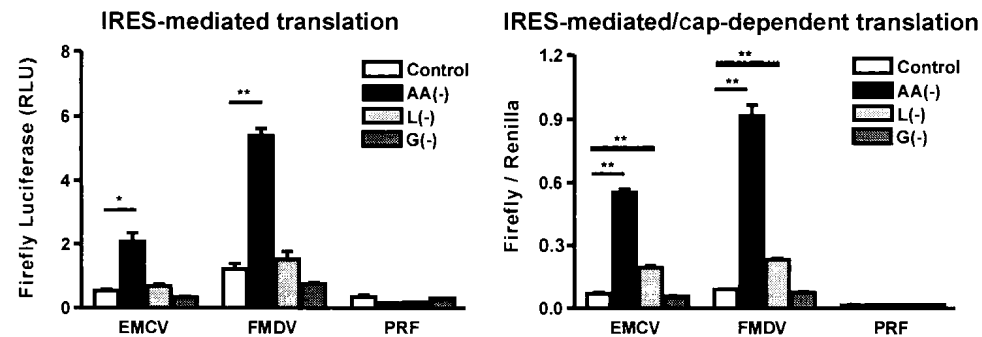


Figure 5.4B-D

IRES reporter constructs were treated with LY294002, we found that translation initiated by EMCV and FMDV IRES was not promoted while the ratio of the viral IRES-mediated to cap-dependent translation was significantly increased, which was due to decreased cap-dependent translation and sustained IRES-mediated translation (Figure 5.4B). Similar results were obtained when cells were treated with the mTOR inhibitor Torin1 (Figure 5.4C). We next investigated whether withdrawal of a single amino acid was sufficient to promote viral IRES-mediated translation. Starvation of specific amino acids, such as leucine, has been previously shown to modulate the mTOR pathway and to induce dephosphorylation of 4E-BPs (Crespo & Hall, 2002; Crespo *et al.*, 2002). As shown in Figure 5.4A, leucine starvation but not L-glutamine starvation induced dephosphorylation of 4E-BP1 with a very similar kinetic compared to complete amino acid starvation. However, the promotion of EMCV and FMDV IRES-mediated translation was only observed in starvation of complete amino acid, but not in leucine or L-glutamine starvation (Figure 5.4D). Both complete amino acid starvation and leucine starvation induced significant changes to the ratio of viral IRES-mediated to cap-dependent translation. Taken together, these results suggest that 4E-BP1 dephosphorylation does not promote EMCV and FMDV IRES-mediated translation while it changes the ratio of the viral IRES and cap-dependent translation by inhibiting eIF4E function to initiate cap-dependent translation.

5.2.4 ITAFs modulation by amino acid starvation

In order to investigate whether amino acid starvation changes the binding rate of FMDV

ITAF(s), we isolated FMDV IRES binding proteins by RNA affinity chromatography from cells treated with or without amino acid starvation. Cellular extracts of NIH3T3 cells cultured in control or amino acid-starved conditions were incubated with FMDV IRES RNA-oligo dT beads complexes. Pull-down samples without FMDV IRES RNA were included as negative control. RNA associated proteins were eluted and run on SDS-PAGE and subjected to silver staining and western blot analysis for FMDV IRES known ITAFs (hnRNPI and Ebp1) (Figure 5.5). As seen in the no RNA control sample of figure 5.5A, we observed non specific binding of proteins to the beads in the absence of FMDV IRES RNA, suggesting that further modification of the experimental protocol is required. Nevertheless, more proteins were isolated with the FMDV IRES RNA-oligo dT beads (lanes: Control and AA (-)) which suggests the presence of the FMDV ITAFs in the pull down proteins. On the silver staining gels, we did not observe any differential patterns of the pull-down proteins isolated from control cells and amino acid starved cells. To further determine whether FMDV ITAFs were present in the pull down samples, we conducted western blot analysis against hnRNPI and Ebp1 (Figure 5.5B and C). Both hnRNPI and Ebp1 were detected only in the FMDV IRES RNA-pull down samples but not in RNA(-) control samples. However, the amount of hnRNPI and Ebp1 pulled down by FMDV IRES RNA was similar between cell lysates derived from control cells and amino acid starved cells, suggesting that amino acid starvation did not affect the binding of these ITAFs to FMDV IRES.

Figure 5.5: Isolation of proteins interacting with FMDV IRES by RNA affinity

chromatography assay. RNA corresponding to FMDV IRES was incubated with protein extracts from NIH3T3 cells incubated in control or amino acid-starved (AA-) media. The assay was performed also in the absence of RNA as a control for proteins unspecific binding. RNA-protein complexes were isolated using magnetic beads. Pull down proteins were separated by SDS-PAGE gels and **A)** silver stained or transfer to membranes for western blot analysis with **B)** hnRNPI or **C)** Ebp1 specific antibodies.

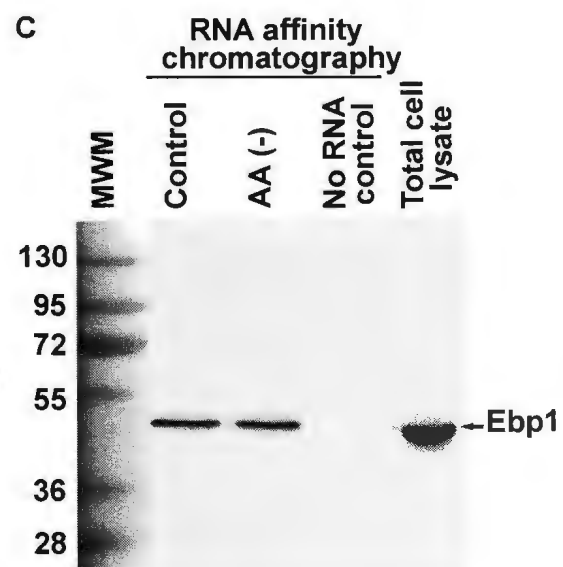
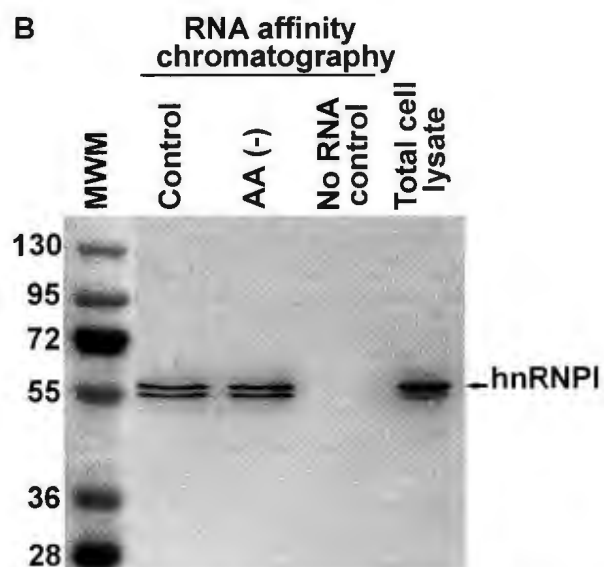
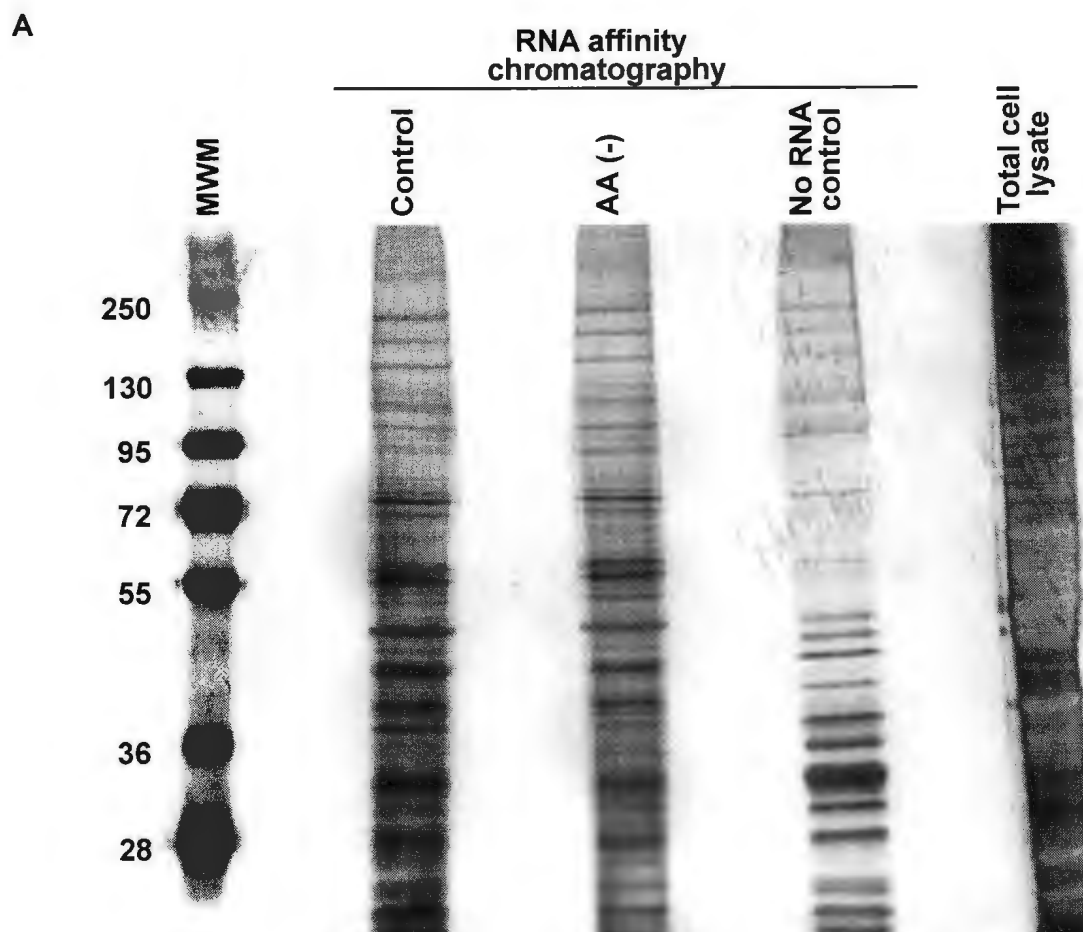


Figure 5.5

CHAPTER 6

DISCUSSION

6.1 Comparison of viral and cellular IRES elements efficiency in physiological condition

6.1.1 Use of VCIP IRES element in bicistronic vectors to improve their efficiencies

IRES elements ability to promote internal initiation of translation has been exploited to achieve co-expression of multiple genes. Multi-cistronic expression vectors contain an IRES element as a linker between genes, which avoids suppression of alternative promoters, a common problem when two or more genes are included into a single vector under different promoters (Allera-Moreau *et al.*, 2006; Allera-Moreau *et al.*, 2007; Delluc-Clavieres *et al.*, 2008; Li *et al.*, 2007; Morgan *et al.*, 1992). In these multi-cistronic vectors, the primary mRNA transcript is modified by a 5'-cap structure, similar to endogenous mRNA transcripts, to allow translation of the first gene by a cap-dependent, ribosome scanning mechanism. Translation of subsequent genes is accomplished by the direct recruitment of ribosomes to the IRES in a cap-independent manner. Although these vector designs ensure multiple genes are expressed from a single transcription unit (Bouabe *et al.*, 2008; Martinez-Salas, 1999; Wang *et al.*, 2005b; Wong *et al.*, 2002), high expression levels of genes downstream of the IRES is not always achieved.

In Chapter 3, to compare cellular and viral IRES activity in physiological conditions, we conducted *in vitro* and *in vivo* screening of 13 IRES elements. Importantly, we found that VCIP IRES was the only IRES to significantly increase translation initiation of the downstream gene compared to EMCV IRES in all the cell lines tested (Figure 3.2), as well as in mice (Figure 3.4). Furthermore, we confirmed the previous

report that introduction of a VCIP IRES does not affect the stability of bicistronic mRNA, introduce a cryptic promoter, or result in alternative mRNA transcripts being produced (Figures 3.5 and 3.6) (Blais *et al.*, 2006).

VCIP, also known as phosphatidic acid phosphatase-2b (PAP2b) and human lipid phosphate phosphohydrolase-3 (LPP3), plays critical roles in many cellular functions. VCIP is ubiquitously expressed in tissues from mouse and human origins (Kai *et al.*, 1997; Shmueli *et al.*, 2003; Su *et al.*, 2004), and is essential during mouse embryo development since VCIP knockout embryos die between embryonic days 7 to 9.5 (Escalante-Alcaide *et al.*, 2003). VCIP has lipid phosphatase activity and converts phosphatidic acid (PA) into diacylglycerol, a second messenger implicated in lipid metabolism. VCIP is also known as a pro-angiogenic protein since addition of anti-VCIP antibody significantly blocked capillary morphogenesis of endothelial cells (Blais *et al.*, 2006; Humtsoe *et al.*, 2003; Wary & Humtsoe, 2005). Because the efficiency of IRES-mediated translation is highly dependent on the cellular availability of ITAFs (Martinez-Salas, 1999; Ngoi *et al.*, 2004), and considering the important biological roles of VCIP, it is likely that the expression levels and activity of VCIP's ITAFs are consistently high in a broad range of cell types. Interestingly, VCIP IRES activity increases by 20 fold under hypoxic stress compared to normal cell culture conditions (Blais *et al.*, 2006). Since it is well documented that the microenvironment of solid tumors is usually hypoglycemic and hypoxic (Carmeliet & Jain, 2000; Graeber *et al.*, 1996), this particular feature of VCIP IRES may be useful for cancer gene therapy protocols.

We found that some IRES elements demonstrate cell line-specific protein expression. For example, translation mediated by c-myc IRES was more efficient than EMCV IRES in Huh7 and MRC-5 cell lines, while Rbm3 IRES-dependent translation was significantly different in MEF as well as in Huh7 cells (Figure 3.2). These results are in concordance with Wong *et al.* who demonstrated higher gene expression mediated by c-myc IRES compared to EMCV IRES in KB-3-1 and N2a cells but not in HEK293 cells (Wong *et al.*, 2002). This observation warrants further study as this may enable tissue-specific expression of a gene, which may be important for gene therapy applications.

It needs to be mentioned that the sequence of the EMCV IRES currently used in expression vectors has been modified from that of the wild type EMCV IRES (Bochkov & Palmenberg, 2006; Hennecke *et al.*, 2001). During the establishment of expression vectors with an EMCV IRES, the native EMCV IRES has been modified to create restriction enzyme sites to allow ligation of multiple reading frames in these vectors. The native EMCV AUG has also been removed in some of commercial vectors. The modified EMCV IRES sequence has been shown by Bochkov and Palmenberg to reduce protein expression by up to 10-fold compared to the native sequence (Bochkov & Palmenberg, 2006). Blais *et al.* demonstrated that the VCIP IRES element spans 140 to 380 nucleotides within the 568 nucleotides length of the VCIP 5'UTR used in this study (Blais *et al.*, 2006). However, deletion of the first 140 nucleotides resulted in abolishment of VCIP IRES activity while translation initiation was partially inhibited in the absence of the 380 to 568 nucleotides region compared to the full length 568 nucleotides VCIP IRES element. These data suggest that VCIP IRES activity depends on optimal IRES secondary

structure affected by the upstream and downstream sequences as well as the native VCIP AUG. Another possibility might be that there are ITAF binding sites within the deleted regions and absence of these sequences reduce or abolish the IRES activity. Therefore, it is likely that the full length 568 nucleotides VCIP IRES element is required in expression and gene therapy vectors to achieve the most efficient protein expression.

In summary, expression vectors harboring a VCIP IRES consistently showed greater efficiencies of protein expression in both cell culture (48 to 538-fold higher) and in animal models (23-fold higher) compared to EMCV IRES. The introduction of the VCIP IRES into the bicistronic vector did not cause modification of the bicistronic transcript such as splicing, cryptic promoter generation or instability of the vector mRNA. These results warrant further studies to develop expression vectors containing the VCIP IRES for improved biomedical research and gene therapy applications.

6.2 Promotion of viral IRES-mediated translation under amino acid starvation

Virus infection induces cellular stress conditions leading to activation of cellular stress response pathways. IRES-mediated translation has been considered as one viral strategy to ensure efficient production of viral proteins under cellular stress conditions where host cap-dependent translation is severely impaired. In Chapter 4, we used bicistronic reporter constructs to screen the efficiency of viral IRES-mediated translation under different cellular stresses. We found amino acid starvation to be the only cellular stress tested that promoted firefly luciferase IRES-mediated translation by EMCV and FMDV IRES elements (Figure 4.1). In Chapter 5, we further investigated the involvement of three

major stress response pathways modulated by amino acid starvation, mTOR-4E-BPs, GCN2 and eIF2B, in the promotion of the viral IRES-mediated translation. We found that knockdown of 4E-BP1 impaired the promotion of the viral IRES-mediated translation during amino acid starvation (Figure 5.1) while GCN2 and eIF2B ϵ were seemingly not involved (Figure 5.2 and 5.3). This is the first study to systemically investigate translation efficiency initiated by viral IRES elements under cellular stresses and furthermore, to identify the cellular machinery involved.

Posttranslational modulation of 4E-BPs has been found to occur during infection with different viruses. EMCV and PV infection increased levels of dephosphorylated 4E-BPs, which sequester eIF4E contributing to the shutoff of host protein synthesis (Gingras *et al.*, 1996). Since eIF4E, which is responsible for the recognition of the cap-structure, is not required for EMCV and PV IRES-mediated translation, production of viral proteins can be achieved in cells with dephosphorylated 4E-BPs. The dephosphorylation of 4E-BPs is also induced at the early stage of vesicular stomatitis virus (VSV) infection (Connor & Lyles, 2002). VSV mRNA is capped at its 5' end, however, it was suggested that translation of viral mRNA can still be initiated with very low amount of eIF4E due to the short length of the 5' UTR (Connor & Lyles, 2002). In contrast, other viruses such as adenovirus are known to promote phosphorylation of 4E-BPs to enhance cap-dependent translation for viral protein synthesis (Gingras & Sonenberg, 1997; Huang & Schneider, 1991). Svitkin *et al.* demonstrated that overexpression of 4E-BP1 promotes EMCV IRES-mediated translation, suggesting that eIF4E availability regulates translation efficiency of EMCV IRES element by controlling the switch between the rate of cap-dependent

translation and IRES-mediated translation (Svitkin *et al.*, 2005). Similar to these previous findings, we also observed the involvement of 4E-BP1 in the promotion of EMCV and FMDV IRES-mediated translation. The knockdown of 4E-BP1 impaired the promotion of EMCV and FMDV IRES-mediated translation under amino acid starvation (Figure 5.1). Interestingly, however, we observed that amino acid starvation did not increase the efficiency of translation initiation of the other viral IRES elements (HCV, HRV and PV), which, similarly to EMCV and FMDV IRES, do not require eIF4E for their translation initiation (Ohlmann *et al.*, 1996; Pestova *et al.*, 1996b). In addition, the other cellular stresses we tested in this study, which are also known to induce 4E-BPs dephosphorylation (Hara *et al.*, 1998; Kim *et al.*, 2002; Rong *et al.*, 2008), did not promote EMCV and FMDV IRES-mediated translation. Therefore, these indicate that the eIF4E availability modulated by dephosphorylation of 4E-BP1 may not be the sole mechanism for promotion of EMCV and FMDV IRES-mediated translation under amino acid starvation. More importantly, the promotion of the viral IRES-mediated translation was not observed under 4E-BP1 dephosphorylation induced by the PI3K inhibitor, mTOR inhibitor or leucine starvation (Figure 5.4).

Here, we propose three possible mechanisms of how 4E-BP1 is involved in the promotion of EMCV and FMDV IRES-mediated translation under amino acid starvation. First, dephosphorylation of 4E-BP1 is essential, but not sufficient, since there are also other cellular mechanisms required for the promotion of the viral IRES-mediated translation. For example, amino acid starvation increases binding of ITAFs to CAT-1 IRES element and efficiency of its translation initiation (Majumder *et al.*, 2009).

Therefore, amino acid starvation may modulate the ITAFs binding to the IRES elements in addition to 4E-BP1 dephosphorylation, while treatment with PI3K/mTOR inhibitor or leucine starvation only dephosphorylates 4E-BP1. Secondly, specific phosphorylation status of 4E-BPs may be required for the promotion of the viral IRES-mediated translation. There are multiple phosphorylation sites on 4E-BP1, which are differently regulated (Gingras *et al.*, 1999; Gingras *et al.*, 2001; Herbert *et al.*, 2002). An mTOR inhibitor, rapamycin, can inhibit phosphorylation of Thr69 and Ser65 of 4E-BPs (Beugnet *et al.*, 2003). In contrast, phosphorylation of Thr37 and Thr46 of 4E-BP1 is amino acid-dependent, but rapamycin insensitive (Beugnet *et al.*, 2003; Gingras *et al.*, 1999; Wang *et al.*, 2005a). Therefore, a specific phosphorylation status of 4E-BP1 may be essential for the promotion of the viral IRES-mediated translation under amino acid starvation. Finally, 4E-BP1 may regulate the viral IRES-mediated translation via a downstream element distinct from eIF4E. Although 4E-BP1 exerts a broad range of cellular functions such as translation, regulation of cell growth and oncogenesis (Dowling *et al.*, 2010; Petroulakis *et al.*, 2009; She *et al.*, 2010), the regulation of eIF4E by dephosphorylated 4E-BP1 is currently the only known function of 4E-BP1. However, there may be an unidentified downstream roles played by 4E-BP1 that are responsible for the promotion of the viral IRES-mediated translation.

We found that FMDV IRES-mediated translation was promoted under amino acid starvation in NIH3T3, HCT116 and Vero cells, but not in Huh7 cells, suggesting a cell-type specific event (Figure 4.3). Different cellular components such as stress response pathways, 4E-BP and ITAFs are required for the promotion of viral IRES-mediated

translation. The activity of the cellular components varies depending on cell origin and degree of transformation, which may contribute to the differences seen between cell types regarding IRES-mediated translation under amino acid starvation. IRES-mediated translation is a critical step in the replication cycles of picornaviruses and HCV.

Different IRES elements require a distinct subset of ITAFs for their translation initiation, which contribute to cell type specific translation of genes (Holcik & Sonenberg, 2005; Sarnow, 2003). We sought to determine whether amino acid starvation modulates the binding status of FMDV ITAFs using RNA affinity chromatography. Recently, using the same method, Pacheco *et al.* identified 21 RNA binding proteins interacting with FMDV IRES (Pacheco *et al.*, 2008). We used full length of FMDV IRES instead of independent IRES domains used in the previous study. As shown Figure 5.5, we have successfully pulled- down PTB and Ebp1 proteins, well known ITAFs for FMDV IRES. However, there were no differences between cells treated with amino acid starvation or control media in the amount of these proteins pulled down. It is possible that amino acid starvation leads to some post translational modification of specific ITAFs affecting its activity but not its binding. The protein samples will be further examined for other FMDV ITAFs such as heterogeneous nuclear ribonucleoprotein K (hnRNPK), Ras-GTPase-activating protein (G3BP) or heterogeneous nuclear ribonucleoprotein U (hnRNPU). The ITAFs binding to an IRES element is dependent on their expression levels, posttranslational modification and translocation under stress condition. The RNA affinity chromatography could identify the binding changes induced by expression and posttranslational modifications or changes in the cytoplasmic amount of binding proteins.

Translocation should be further determined by analysis of nuclear versus cytoplasmic levels of protein in control and amino acid starved conditions.

In chapters 4 and 5, we identified 4E-BP1 as a cellular factor that increases translational initiation from EMCV and FMDV IRES elements by screening the relationship between cellular stresses and viral IRES-mediated translation. Our findings are essential to understand the replication strategies of viruses containing an IRES element and to identify targets for the development of antiviral drugs.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

1) The efficiency of translation initiation in physiological conditions varies among IRES elements. The IRES elements also exhibit cell type-dependent translational activity.

In this study, I compared the efficiency of cellular and viral IRES elements for their translation initiation in physiological conditions using bicistronic reporter constructs. I demonstrated that efficiency of the IRES elements on their translation initiation in physiological conditions varies significantly in the cell lines investigated. In addition, the same IRES element demonstrated more efficient translation initiation in certain cell lines. It is most likely due to different availability of ITAFs among the cell lines.

2) VCIP IRES directs translation more efficiently than any other IRES I tested in vitro and in vivo.

There are IRES elements that demonstrated higher efficiency of translation initiation than EMCV IRES element, which is most commonly used for gene co-expression in bicistronic vectors. Among them, VCIP IRES consistently demonstrated higher efficiency than EMCV IRES element in the cell lines investigated (48 to 538-fold higher), as well as in animals (23-fold higher). Therefore, I believe that VCIP IRES element has a great potential to improve the efficiency of bicistronic vectors used in gene therapy application and biomedical research.

3) Amino acid starvation promotes EMCV and FMDV IRES-mediated translation.

Translation of cellular mRNA containing an IRES element can be promoted under cellular stress conditions. However, it remained to be studied whether viral IRES-mediated translation can be modulated by cellular stresses. By screening of translation efficiency initiated by viral IRES elements under different cellular stress conditions using bicistronic reporter constructs, I found that amino acid starvation was the only cellular stress tested that promoted the translation rate initiated by viral IRES elements. Since the promotion was observed in translation initiation by EMCV and FMDV IRES elements, but not in that by other viral IRES elements, amino acid starvation may activate a specific cellular stress response pathway(s) involved in regulating EMCV and FMDV IRES-mediated translation.

4) 4E-BP1 is involved in the promotion of EMCV and FMDV IRES-mediated translation under amino acid starvation.

Amino acid starvation inhibits cap-dependent translation by inducing dephosphorylation of 4E-BP, which is downstream of one of the key cellular stress response pathways that regulate protein synthesis (Kimball, 2001). We demonstrated that knockdown of 4E-BP1 impairs the promotion of the viral IRES-mediated translation during amino acid starvation. Interestingly, however, the promotion of the viral IRES-mediated translation was not observed under 4E-BP1 dephosphorylation induced by the PI3K inhibitor, mTOR inhibitor or leucine starvation. These results suggest that 4E-BP1 is required for the

promotion of EMCV and FMDV IRES-mediated translation, but that dephosphorylation of 4E-BPs as mediated by PI3-K inhibitor, leucine starvation or mTOR inhibitor is not sufficient.

7.2 Future directions

1) Further analysis of ITAFs of EMCV and FMDV IRES elements modulated during amino acid starvation.

To identify the downstream pathway involved in the promotion of the viral IRES-mediated translation, I believe that it is the best approach to analyze the binding status of the ITAFs during amino acid starvation. I have established the pull-down system of ITAFs by RNA affinity chromatography and was able to confirm the presence of FMDV IRES ITAFs in the pull-down products by western blotting analysis while many nonspecific bands were found in silver staining gels. In the future I can further optimize the protocol to reduce nonspecific binding proteins, and analyze differences in binding of ITAFs to the IRES element in control and amino-acid starved cells. Techniques such as mass spectrometry or quantitative mass spectrometry, like mass differential tags for relative and absolute quantification (m-TRAQ), will be used to identify the ITAFs that differentially bind to the viral IRES elements under amino acid starvation. Once the ITAFs are identified, I will determine their postranslational modification or translocation induced by amino acid starvation.

2) Identify phosphorylation status of 4E-BP required for the promotion of the viral IRES-mediated translation.

We found that phosphorylation induced by mTOR inhibitors and leucine starvation did not promote EMCV and/or FMDV IRES-mediated translation. As discussed above (page 101-102), it is possible that these treatments result in different phosphorylation status of 4E-BPs from that induced by amino acid starvation. To study this, I will conduct experiments using 4E-BP1 mutant constructs in which individual phosphorylation sites have been mutated (kindly provided by Dr. N. Sonenberg's lab) and determine whether amino acid starvation induces a particular 4E-BP1 phosphorylation state responsible for the promotion of EMCV and FMDV IRES-mediated translation.

3) Determine whether the identified pathway is involved in regulating picornavirus infection.

My thesis focuses on studying the mechanisms of translational initiation by an IRES element, which is one of critical steps in certain viral replication cycles. Bicistronic reporter constructs are excellent tools to screen IRES-mediated translation under cellular stress as well as to identify the cellular stress response pathways regulating the viral IRES translation initiation. However, I believe that it is essential to determine the roles of the identified cellular pathway (4E-BP pathway) in competent replication cycles. Since the EMCV replication system has been established in Hirasawa laboratory, it is of interest to conduct gain- and loss-in function experiments of the 4E-BP in EMCV-infected cells. A

better understanding of the regulation of IRES-mediated translation would eventually allow the development of new therapies against virus infection.

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